CREATING DELETION STRAINS TO STUDY THE ROLE OF THE ARGONAUTE AND DICER GENES IN RNA INTERFERENCE IN CRYPTOCOCCUS NEOFORMANS

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DEDICATION

I dedicate this work to my family. To my loving wife Traci and my beautiful daughter Addison: Thank you for serving as an inspiration for me.

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ABSTRACT

CREATING DELETION STRAINS TO STUDY THE ROLE OF THE ARGONAUTE AND DICER GENES IN RNA INTERFERENCE IN CRYPTOCOCCUS NEOFORMANS

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Cryptococcus neoformans is a spherical, encapsulated, basidiomycetous yeast and the causative agent of cryptococcosis, a form of meningitis that affects the central nervous system of immunocompromised individuals (immunocompromised means patients with compromised immune systems). Since the 1980's and the emergence of the AIDS epidemic, much study has been concentrated on this fungus because cryptococcosis is 100% fatal in untreated patients. Even with treatment, the condition does not always decrease in severity, and no major advancements in antifungal drugs have been made in a decade. Recently, *Cryptococcus* has been shown to possess the necessary machinery for RNA interference (RNAi). RNAi is a method of post-transcriptional gene silencing that may increase cryptococcal survival within mammalian hosts by controlling gene expression at various stages of the life cycle through heterochromatin and euchromatin rearrangement. RNAi was first described in *Caenorhabditis elegans* in 1998 by Andrew Fire and his colleagues, and is best studied in *Drosophila melanogaster*. The pathway includes **argonaute** and **dicer** proteins that aid in highly specific degradation of mRNA

in vivo. To date, no organism has been shown to utilize RNAi as a factor for virulence, nor should it be thought of as such; however, this pathway may indeed play a role in the formation of certain virulence factors for *Cryptococcus*, including the transcription of heat stress response genes required for thermotolerance, oxidative stress response genes such as those required for melanin and mannitol biosynthesis, as well as the formation of the polysaccharide capsule. Two separate, yet equally effective methods were used in order to create deletion constructs of the *AGO1* and *AGO2* genes. Overlap PCR and traditional cloning methods were employed. While overlap PCR was optimized, molecular cloning techniques were also used to create the complementation constructs for the above genes.

Through overlap PCR, this study resulted in the creation of deletion constructs for the *AGO1* and *AGO2* genes, which are thought to play a role in RNAi. This study also resulted in the creation of a complementation construct for the *ago1* deletion. These constructs can be biolistically inserted into *C. neoformans* to produce the corresponding deletion strains. Once these strains are produced, future experiments will include many phenotypic studies between them and other known strains, as well as an eventual murine virulence study.

INTRODUCTION

Cryptococcus neoformans is a spherical, encapsulated, basidiomycetous yeast, and the causative agent of cryptococcosis (Buchannan & Murphy, 1998). Until recently, two varieties of *C. neoformans* were recognized: *C. neoformans* var. *neoformans* and *C. neoformans* var. *gattii* (Mitchell & Perfect, 1995). However, *C. neoformans* var. *gattii* is now considered a separate species (*C. gattii*), and a new variety, *grubii* has been assigned to *C. neoformans*, citing differences in phenotype, biochemistry, epidemiology, and other factors (Jaine & Fries, 2008). The two species differ from each other in terms of capsule epitopes (serotype), biochemistry, epidemiology as well as geographical distribution and habitat preference (Mitchell & Perfect, 1995; Jaine & Fries, 2008). This cosmopolitan fungus is widespread throughout the geography of the planet, with *C. neoformans* found worldwide, and *C. gattii* emergent in tropical and sub-tropical environments.

Cryptococcus gattii is often found in river forest gums (*Eucalyptus camaldulensis*) and forest red gums (*Eucalyptus tereticornis*) (Casadevall & Perfect, 1998). *Cryptococcus neoformans* was first isolated from fermenting peach juice in 1894 by Sanfelice. No other environmental isolates were recorded until 1951 when Emmons described *C. neoformans* in soils containing avian guano (Emmons, 1951), although *C. neoformans* was found inside skin lesions prior to 1951 (Mitchell & Perfect, 1995). In fact, *C. neoformans* is closely associated with soils containing avian excreta, more specifically, pigeon (*Columba livia*) guano, and soils which are near avian nesting sites.

However avian species are not at risk for a cryptococcal infection (Litvintseva et al., 2005, Nielsen et al., 2007). In addition to soil and pigeon waste, *C. neoformans* has also been isolated from the air and other unusual places like bat caves, cockroaches, rabbit housings, and even bagpipes. *Cryptococcus neoformans*, like *C. gattii*, has also been associated with species of *Eucalyptus* trees. *Cryptococcus neoformans* has also been isolated from soils in Africa, Asia, Europe, North and South America, the Caribbean, and the Pacific Islands (Casadevall & Perfect, 1998). It is, therefore, global in distribution and quite ubiquitous in nature.

Serotypes

C. neoformans has a prominent capsule that surrounds the organism. This structure can be seen quite clearly upon staining the cells with India ink (Maxson et al., 2006), and serves as the primary diagnostic tool for central nervous system infections (Bindschadler & Bennet, 1968). Capsules of different cryptococcal varieties differ subtly in structure, and these differences can be identified by the discriminatory binding of antibodies to these different strains. Based on the various antibody responses generated, *C. neoformans* strains have been divided into serotypes that correlate well to the species nomenclature. There are five different serotypes: A, B, C, D, and AD hybrids. *Cryptococcus neoformans* var. neoformans can possess capsular serotypes D or the AD hybrid; while *C. neoformans* var. *grubii* is the sole possessor of serotype A (Jaine & Fries, 2008). *Cryptococcus gattii* may possess either serotype B or C. They tend to infect immunocompetent hosts and usually carry a worse prognosis (Mitchell & Perfect, 1995).

Of the five previously described serotypes, serotype A appears to be the most predominant, causing 99% of all cryptococcal infections in AIDS patients and greater than 80% in non-AIDS patients (Mitchell & Perfect, 1995). Although *C. gatti*, which expresses serotypes B and C, are capable of pathogenesis within hosts with a competent immune system, infection is infrequent due to their limited geography, and thus only affect small populations in Africa and Australia (Litvintseva & Thakur, 2005). Serotype A is more virulent and more common than Serotype D (Cherniak et al., 1980, Jaine & Fries, 2008).

Mating System

Cryptococcus neoformans is considered bipolar, meaning that the organism exists sexually as two mating types – mating type ***a*** (*MAT* ***a***) and mating type **α** (*MAT* **α**). The *MAT* locus of the genome governs the sexual cycle of yeast and yeast-like fungi by establishing cell-type identity, and differs in DNA sequence between cells of opposite mating type. In *C. neoformans,* for sexual reproduction to be successful, mating cells must possess the opposite allele (i.e., **α** with ***a***); although recent work by Joseph Heitman et al. has shown that upregulation of the *CPR2* gene may allow for **α**/**α** matings. This may explain the Vancouver Island breakout in the late 1990's (Hsueh, Xue, and Heitman, 2009) where *C. gattii* infections rose to as much as 20 per million per year, twice the normal rate. Some fungi are capable of switching mating types (homothallic); however, *C. neoformans* is heterothallic, meaning no mating type switching occurs. The *MAT* locus is not only involved in mating type identification, but has also been linked to virulence. *Cryptococcus neoformans MAT* ***a*** is more prevalent in both environmental and

clinical settings and is more virulent than *MAT* ***a*** types, in some serotypes (Fraser & Heitman, 2003; Neilsen, Marra, et al., 2005).

Infection

Cryptococcus neoformans is one of the few fungi that are capable of causing disease in immunocompromised mammalian hosts. Patients with T-cell deficiencies are especially at risk of contracting a cryptococcal infection (Buchanan & Murphy, 1998). Patients with HIV/end-term AIDS, cancer patients who are receiving aggressive radiation therapy or chemotherapy as treatment, as well as transplant patients who are prescribed post-transplant immunosuppressant steroids and individuals with congenital immune system defects are at a higher risk for cryptococcal infections (Waterman et al., 2007). *Cryptococcus* enters the body through the lungs where it can establish a primary infection (Blanco & Garcia, 2008). If the primary pulmonary infection is not held in check by the host immune system, the infection may spread to other organs and organ systems, especially the brain and the central nervous system. *Cryptococcus neoformans* infects several organ systems of mammalian hosts, including the central nervous, cardiac, and respiratory systems. Brain infections lead to cryptococcal meningitis, or cryptococcosis (Traynor & Huffnagle, 2001; Aguirre et al., 2004; Chen et al., 2007). Heart disease can also be associated with *C. neoformans*. Meningoencephalitis has a grimmer prognosis and is associated more closely with HIV/AIDS patients, whereas cryptococcal pneumonia is more often found in transplant patients and cancer patients (Buchanan & Murphy, 1998).

If untreated, cryptococcal meningoencephalitis is 100% fatal, and cryptococcal infections are seemingly non-responsive to even the most effective antifungal drugs if the

host lacks an adequate T-cell-mediated immune response. Cell-mediated immunity is essential for recovery in healthy individuals (Mitchell & Perfect, 1995). Current FDA approved antifungals such as caspofungin are ineffective against a cryptococcal infection. These echinocandins target the cell wall of the fungus and are the only developments in antifungal drugs in the last decade (Odds, Brown, & Gow, 2003). Thus, there is a dire need for novel antifungals that can be used as treatment options for cryptococcosis. The HIV/AIDS pandemic, aggressive cancer treatments and an increase in organ transplant surgeries have fueled the increase in cryptococcosis cases world-wide (Mitchell & Perfect, 1995). Because of this, and more importantly because the fungus has a high rate of mortality in immunocompromised patients, research on this organism has surged since the 1980's.

***Cryptococcus neoformans* and Immunity**

Given the prevalence of *C. neoformans*, cryptococcosis is a relatively rare occurrence, suggesting that many more individuals come in contact with this organism than actually develop systemic disease. Hosts with healthy cell-mediated immunity and a normal level of phagocytes have an effective defense against the fungus, and it has been shown that most healthy individuals possess antibodies to epitopes located on the cell wall of *C. neoformans* (Bindschadler & Bennett 1968; Keller et al., 1994). *Cryptococcus neoformans* infects predominately via the lungs because normal innate barrier-type defenses (skin, mucous membranes) are successful in blocking the pathogen's access to the interior. Regarding defense, cell mediated immunity (CMI) can recognize the different serotypes of *C. neoformans* and is needed for an effective immune response for many mycoses (Blanco & Garcia, 2008).

Cell mediated immunity has two branches, T_{H-1} and T_{H-2}. The T_{H-1} branch of CMI is the mechanism responsible for clearance of a fungal pathogen such as *C. neoformans*. This is because T_{H-1} up-regulates interferon-gamma, increasing cellular immunity and phagocyte (macrophage) activation, whereas T_{H-2} up-regulates interleukins 3 and 4 (IL-3 and IL-4), increasing antibody production (Blanco & Garcia, 2008; Traynor & Huffnagle, 2004). Macrophages and neutrophils are the two major types of phagocytic cells responsible for clearing a cryptococcal infection (Clemmons et al., 2000). All of this relates to the pathogenicity of *C. neoformans* because CMI is dependent upon cluster differentiation 4 positive ($CD4^+$) T-cells (Kwon-Chung et al., 2000; Aguirre et al., 2004). Once macrophages process *C. neoformans*, antigens are presented via MHC-II and $CD4^+$ T-cell receptors recognize these antigen fragments, produce cytokines to recruit more macrophages, and proliferate, creating a full blown immune response (Kwon-Chung et al., 2000). HIV/AIDS patients are susceptible to a cryptococcal infection due to a low CD^{4+} T-cell count that cripples the immune system resulting in great difficulty in clearing such an infection. However, there are unique features of this fungus that allow it to cause systemic infection since most fungal infections are superficial.

Virulence Factors

Virulence factors are simply any physiological or biochemical trait that increases an organism's ability to cause infection. *Cryptococcus neoformans* has several different virulence factors. The severity of disease developed by a given host is not only reliant on the organism's virulence but also the state of the host's immune response status (i.e., immunocompromised or immunosuppresed vs. immunocompetent). Recognized virulence factors of *C. neoformans* include thermotolerance, melanin production,

mannitol synthesis, and the polysaccharide capsule (Buchanan & Murphy, 1998). Thermotolerance is perhaps the most simple virulence factor of any pathogen. Without the ability to survive at body temperature (37ºC), *C. neoformans* would be incapable of causing disease. Calcineurin A is a catalytic subunit used as a stress response in yeast, and must be present in *C. neoformans* if the fungus is to survive, proliferate, and cause disease (Mitchell & Perfect, 1995).

Melanin synthesis also plays a role in the ability of *C. neoformans* to cause disease. Melanin production protects the fungus from oxidative reactions found within macrophages, allowing them to survive these types of environments. However, *C. neoformans* is not capable of producing the precursors (i.e., dihydroxyphenols) of melanin, and must obtain these molecules from the environment. The brain is rich in dihydroxyphenylalanine (DOPA), a type of dihydroxyphenol, and the fungus utilizes it to produce melanin (Buchanan & Murphy, 1998).

Mannitol synthesis may also play a role in *C. neoformans* virulence. The effects of mannitol are three-fold; mannitol may increase resistance to free oxygen radicals within the host (produced within macrophages via oxidative burst), as well as decreasing heat and osmotic stress (Buchanan & Murphy, 1998).

High molecular weight polysaccharides on the surface of the capsule determine the serotype, and thus determine virulence, as some serotypes are more virulent than others. Serotype A is more virulent than serotype D (Cherniak et al., 1980, Jaine & Fries, 2008) and is only found in *C. neoformans* var. *grubii*, leaving serotypes D and the AD hybrid as the sole serotypes of *C. neoformans* var. *neoformans* (Jaine & Fries, 2008). Also, acapsular *C. neoformans* mutants have a greatly reduced degree of virulence in

relation to wild-type *C. neoformans* fungi with a functional capsule (Fromtling et al., 1982). Neutrophils, monocytes, and macrophages are more successful in killing acapsulated fungi, because the capsule protects the fungus from phagocytosis, thus decreasing antigen presentation to T-cells, and reducing the overall immune response (Buchanan & Murphy, 1998). Also, wild type encapsulated *C. neoformans* does not stimulate the production of proinflammatory cytokines such as TNF-α, IL-1β, and IL-6 as effectively as acapsular mutants (Levitz et al., 1994; Vecchiarelli et al., 1995; Delfino et al., 1997).

RNA Interference

Cryptococcus neoformans is capable of performing RNA interference (RNAi). RNAi is a well-studied method of post-transcriptional gene silencing (Dykxhoorn et al., 2005) that was first observed in petunias, but could not be explained. RNAi was formally described by Andrew Fire and his colleagues in *Caenorhabditis elegans*. The molecular mechanism was determined using genetic studies of *Drosophila melanogaster*. In 1990, Carolyn Napoli and her colleagues were attempting to create an aesthetic variety of petunia that was dark purple in color (Napoli et al., 1990). To do this, they created transgenic plants containing multiple copies of the chalcone synthase gene, an enzyme required for the production of the purple pigment. Upon inflorescence, the flowers were not dark purple; rather, they were white, or contained blotchy patterns of purple and white. The reason for this phenomenon was unknown at the time, although it was hypothesized that some form of post-transcriptional gene silencing, termed 'reversible co-suppression,' was responsible. One year later, Fire and his colleagues were attempting to abrogate gene expression in *Caenorhabditis elegans* via introducing ssRNA

into the nematode (Fire et al., 1991). Later, they were attempting the same goal and used a racemic mixture of sense and anti-sense small interfering RNA (siRNA) as a control. Serendipitously, they found that introducing the sense and anti-sense mixture into *C. elegans* led to the specific degradation of homologous mRNA. They propsosed that the sense and anti-sense strands hybridized *in vivo* and formed dsRNA which was acted on by some catalytic element that successfully silenced the genes. This sequence-specific silencing of genes was evidence of a molecular mechanism they dubbed RNA interference, or RNAi (Fire, Xu, Montgomery, et al., 1998).

Initially, RNAi was thought to be unrelated to other post-transcriptional gene silencing mechanisms in plants and **quelling** in *Neurospora*. However, all such mechanisms utilize dsRNAs that are homologous to the silenced gene (Dykxhoorn et al., 2005). Also, many proteins found in these post-transcriptional gene silencing pathways are highly conserved across the plant, fungi, and animal kingdoms (Fagard et al., 2000; Liu et al., 2003). Since the revelation that RNAi occurs in mammalian cell cultures, researchers have attempted to understand the mechanisms behind RNAi and investigate any therapeutic use (Tuschl et al., 1999; Elbashir et al., 2001; Dykxhoorn et al., 2005).

Although the RNAi pathway is most well described in *Drosophila melanogaster*, the major effectors described below are also found in *Cryptococcus neoformans*. Small interfering RNAs (siRNAs) direct mRNA degradation *in vivo*. siRNAs are short (20-25 nucleotides) double-stranded portions of RNA, which are produced from much longer dsRNA segments. The dsRNA segments are digested by a family of RNase III-like proteins called **dicers**. Dicer proteins are thought to be found in the cytoplasm, and act on dsRNA present in the cytoplasm. dsRNA can be found in the cytoplasm for many

reasons, such as infection by a dsRNA virus, transposable elements, or some experimental dsRNA introduced by researchers. Dicers digest long dsRNA in such a way that produces a dsRNA duplex (20-23 nucleotides in length), leaving an overhang of 2-3 nucleotides on the 3' end of each strand (Okamura et al., 2004). Purified dicer can effectively digest long dsRNA into siRNAs *in vitro*. *In vivo*, dicer forms a heterodimer with R2D2, a dsRNA binding protein, which studies suggest allows the siRNA to be incorporated into the RNA-Induced Silencing Complex (RISC) (Tomari et al., 2004; Liu et al., 2003).

Dicers, R2D2, and siRNAs are not the only effectors in this pathway. The highly conserved family of **argonaute** (AGO) proteins is involved in development of organisms, stem cell fate determination, and the RNAi pathway (Carmel et al., 2002). Argonaute proteins are an essential component to the multi-subunit RISC. These proteins are highly basic in pH (Nagayashiki et al., 2005), making them a prime candidate for RNA binding, as argonaute proteins do bind ribohomopolymers *in vitro* (Kataoka et al., 2001). Argonaute proteins are RNase-H like enzymes that contain two highly conserved domains: the PAZ and PIWI domains. The PAZ domain is thought to be responsible for binding the 2-3 nucleotide 3'-overhangs of siRNAs and thus anchor the siRNA to the argonaute protein (Huafang et al., 2004). The PIWI (RNase-H fold) domain of the Argonaute protein is known to bind RNA, but has an affinity for the 5' terminus (Yuan et al., 2005). Argonaute proteins, then, are necessary for siRNA-directed mRNA cleavage. Grishok and his colleagues (Grishok et al., 2000) have shown that particular genes belonging to the argonaute family of proteins are required for RNAi in *C. elegans*, further suggesting that the argonaute proteins play a large role in the RNAi mechanism. Also, in

2004, Okamura and his colleagues discovered that *Δago2 Drosophila*, flies which lack *AGO2*, a gene that encodes an argonaute protein, do not show siRNA-directed RNAi. This study also determined that Argonaute proteins were, at least in *Drosophila*, required for the integration of siRNAs into the RISC and are in some way related to the unwinding of the siRNA duplex (Okamura et al., 2004). Also in 2004, it was determined that Argonaute proteins are the catalytic elements of RNAi, serving as the endonuclease, also called a **slicer**, which loads the siRNA into the RISC and initiates target mRNA cleavage (Liu et al., 2004). After long dsRNAs are processed by dicer into siRNAs and loaded into the RISC via Argonaute and R2D2, the RISC is targeted toward mRNA in the cytoplasm of the cell. The siRNAs identify the mRNA via complementary base pairing, and the Argonaute protein cleaves the mRNA transcript at the 3' end, preventing translation into protein (Figure 1).

To date, RNAi has not been shown as a requirement for virulence in any organism. It has, however, been shown to affect the chromatin status of chromosomes in certain organisms, which could ultimately lead to altered gene expression, possibly leading to changes in expression of certain proteins required for virulence. Because *C. neoformans* possesses the needed machinery for RNAi, it may play a role in the formation of virulence factors that allow the fungus to infect a mammalian host. The first step in establishing the role of RNAi in *C. neoformans* virulence is to create gene deletions of the major players in the RNAi pathway.

In 2006, a study was performed by Hitoshi Nakayashiki and his colleagues with the purpose of screening fungi for genes with homology to *Drosophila* genes involved in RNAi (Nakayashiki et al., 2006). This study found five gene regions within *C.*

neoformans that possess homology to RNAi genes in *Drosophila* and *Arabidopsis*. Of the five genes identified by Nakayashiki, the current work focuses on four. These include the argonaute (*AGO1* and *AGO2*) genes and the dicer (*DICER1* and *DICER2*) genes. Nakayashiki and his colleagues also determined the loci of these genes, allowing for retrieval. Table 1 gives the descriptions of these loci, including gene size.

The entire DNA sequence of the *C. neoformans* genome (with predicted proteins) is available on the web through The Institute for Genomic Research (TIGR) (www.tigr.org/tdb/fungal). Chromosomal information and contigs are also available through the Saint Louis Bioinformatics Facility (genome.slu.edu). Given these sequences, it is feasible to locate these genes and create knock-out constructs by an overlap PCR method (described below), as well as create complementation constructs for the deletions using molecular cloning techniques.

Overlap PCR

The polymerase chain reaction (PCR) was developed by Kary Mullis in 1983. The eventual Nobel Prize winner in Chemistry (1993) is credited with the development of the PCR used in labs around the world today, although Klepp and his co-workers published a study concerning the replication of short DNA templates using primers and DNA polymerases in 1971. This study received little attention however, paving the way for Dr. Mullis' landmark development that changed the way molecular biology is performed by offering an all-purpose tool for many types of experiments.

The basis of PCR is quite simple. Temperature cycling facilitates the polymerization of DNA *in vitro*. To begin with, the double stranded DNA must be separated into single strands of DNA (a process called melting or denaturation). To do

this, the temperature is raised to approximately 94°C, where the hydrogen bonds between the double helix break, resulting in two single strands of DNA. Once the DNA is "melted," the temperature is lowered in order for primer binding to occur (a process called annealing). The annealing temperature varies between primers, and allows for bonds to be formed between the single-stranded DNA and the single-stranded oligonucleotide primer. The 3' end of the primer offers a hydroxyl group, to which the DNA polymerase adds the next nucleotide in the sequence, ultimately resulting in a nascent DNA strand. However, before the polymerase can polymerize the new DNA, the temperature must be raised to approximately 72°C (a process called extension/elongation), or the optimal temperature for the given enzyme's function. This also removes non-specific binding of primers to DNA sequences of low homology, adding stringency to the procedure. The bases added are complementary to the template DNA strand, thus making a complete DNA double-helix with two strands. The reaction progresses in this manner until the temperature is raised again to 94°C and DNA copy number is doubled after annealing and elongation.

Since the mid 1980's, PCR has been adapted for a myriad of molecular applications. Using a heat-stable DNA polymerase such as the TAQ polymerase (from the bacterium *Thermus aquaticus* found in hot springs of Yellowstone National Park), synthesized oligonucleotide primers, deoxynucleotides, a buffer, magnesium chloride (TAQ polymerase cofactor), and a template strand of DNA, PCR can generate many copies of DNA from that template, allowing for subsequent enzymatic reactions, microarray, *in vitro* transcription, microinjection, or sequencing.

PCR has many modifications that allow it to serve many different purposes within molecular biology, as well as other fields. Researchers can utilize PCR to screen for clones using colony PCR. Viral RNA may be amplified using reverse –transcriptase PCR, allowing the viral genome to be sequenced for further genetic and evolutionary studies. Some versions of PCR vary the temperatures used in order to decrease background noise in the reaction. Hospitals have also adapted PCR for use as a diagnostic tool for detecting certain diseases, such as an HIV infection, or certain well-studied diseases such as tuberculosis (Haldar, et al., 2009). PCR has also been modified to allow for multiple fragments of DNA to be fused together in order to create constructs for gene deletion strains of organisms (Davidson et al., 2002), as well as constructs for making fusion proteins for use in immunological studies and drug targeting (Mannie et al., 2006). This form of PCR, termed **overlap PCR**, has gained much popularity and was employed in this study to achieve gene deletion constructs.

The overlap polymerase chain reaction (OL-PCR) is a PCR-based strategy for rapid gene deletion, and has been demonstrated as a high throughput mechanism for genetic analysis (Davidson et al., 2002). OL-PCR was developed by Davidson et al. for use in *Cryptococcus neoformans*, and they used this molecular tool to disrupt several different genes in order to study their function. The technique consists of two separate rounds of PCR, and has comparable efficacy to more traditional cloning techniques. However, OL-PCR is much faster, and is usually a cheaper option.

During the first round of PCR, individual DNA fragments are amplified using primers that generate small overlapping regions between specific fragments. This allows for fusion of all the fragments in the second round of PCR using primers to the outermost

sequences. Overall, to delete chromosomal genes via OL-PCR, the sequences of the genes to be deleted must be known. In order to successfully create deletion constructs using this method, the flanking regions of the gene must be amplified and fused with a selectable marker. The marker is used to confirm the presence of the construct *in vivo*, and the flanking regions are necessary for homologous recombination to occur.

The purpose of this study was to create strains of *Cryptococcus neoformans* which lack the *AGO1* and *AGO2* genes, as well as the *DICER1* and *DICER2* genes from the genome so that further studies can use these strains to test for the functionality of RNAi and ultimately for virulence. It was hypothesized that RNAi would cease to function within strains of *C. neoformans* which lack either both Argonaute or both Dicer genes, and that ultimately, the absence of RNAi would lead to decreased efficacy of virulence within these strains.

METHODS AND MATERIALS

Determining Gene Regions For PCR

The entire genome of cryptococcal strain JEC21 serotype D with predicted proteins is available from the TIGR Fungal Database (www.tigr.org/tdb/fungal). The loci for the four genes targeted for deletion in this study are published and readily available (Nakayashiki, et al., 2006) (Table 1). Nakayashiki and his colleagues identified the *AGO1* and *AGO2* genes to be located at the CNJ00490 and CNJ00610 loci, respectively, and the *DICER1* and *DICER2* genes at the CNC03670 and CNC03680 loci, respectively. The *AGO* genes are on chromosome 10 of the cryptococcal genome, whereas the *DICER* genes are located on chromosome 3. These loci were entered into TIGR and sequences of the genomic DNA of each gene, the coding region, and the amino acid make-up of the protein were retrieved. The sequences from TIGR were blasted against sequences in the Saint Louis Bioinformatics Facility's (SLBF) database of *Cryptococcus neoformans* genome information available at genome.slu.edu, to retrieve additional sequences necessary to generate the 1.2 kb flanks needed for homologous recombination to occur in *Cryptococcus*. Freeware DNA alignment software, SerialCloner v.1-3 (available from http://serialbasics.free.fr/Serial_Cloner.html) was used to align the sequence of the gene (from TIGR) with the chromosomal sequence (from SLBF). This allowed for the acquisition of the gene sequence and approximately 1.2 kb 5' and 3' flanks. The process above was done for the following genes: *AGO1*, *AGO2*, *DICER1*, and *DICER2*.

Primer Design

Four sets of primers were designed for the *AGO1* and *AGO2* genes, as well as two sets of primers for the *DICER1* and *DICER2* genes. Primer design was facilitated using Primer3 software available from the Massachusetts Institute of Technology at frodo.wi.mit.edu.

Argonaute 1 Knockout Primers

Primer sets were created to amplify three fragments- 1.2 kb regions of the 5' and 3' flanks of the *AGO1* gene, as well as a selectable marker. The 5' Out (IBO264) primer consisted of 25 nucleotides with a calculated melting temperature of 59.7°C; the 5' In (IBO265) primer consisted of 23 nucleotides, and a calculated melting temperature of 57.4°C. A 3' Out (IBO266) primer of 24 nucleotides was constructed as well, having a calculated melting temperature of 56.6°C. The 3' In (IBO267) had a calculated melting temperature of 59.5 and was made up of 21 bases. These primers were complimentary to the template DNA.

The overlap primers for *AGO1* were designed to produce approximately 20 bases of overlap between the gene flanks and the selectable marker. A Selection-3' (IBO270) primer was constructed of 42 nucleotides, where 21 nucleotides were complimentary to the template DNA and the remaining 21 nucleotides were complimentary to the flanks of the neomycin ($G418^R$), nourseothricin (NAT), and hygromycin (HYG) resistance markers. The flanks of the plasmid cassettes for the selectable markers are identical, allowing for a single primer set to successfully amplify them. A Selection-5' (IBO504) primer was designed with 44 nucleotides. Half of the nucleotides corresponded to the 5' terminus of the selectable marker, and the other half corresponded to the 3' terminus of

the 5’ flank, to generate the necessary overlapping region. The 5’-Selection (IBO503) primer was also 44 nucleotides in length, with half of those nucleotides designed to add a 5’flank overlap to the 3’ terminus of the selectable marker DNA for overlap generation. The 3’-Selection (IBO271) primer consisted of 42 nucleotides, half of which complimented the 5’ terminus of the 3’ gene flank, with the other half was complementary to the flanking regions of the three drug resistance markers listed above. All overlap-producing primers (IBO270, IBO271, IBO503, and IBO504) were PAGE purified to ensure primer integrity. All eight *AGO1* knockout primer sequences are shown in Table 2.

Argonaute 2 Knockout Primers

Primer sets were created to amplify the 5’ and 3’ flanks of *AGO2* and selectable markers as with *AGO1*. The 5’ OUT (IBO205) primer consisted of 21 nucleotides with a calculated melting temperature of 55.8°C; the 5’ IN (IBO206) primer consisted of 20 nucleotides, with a calculated melting temperature of 50.0°C. A 3’ OUT (IBO207) primer of 21 nucleotides was constructed to have a calculated melting temperature of 63.2°C. The 3’ IN (IBO208) had a calculated melting temperature of 59.5°C and was made up of 20 bases. These primers were complimentary to the template DNA.

The overlap primers for *AGO2* were designed in the same manner as for *AGO1*. A Selection-3’ (IBO501) primer was constructed of 44 nucleotides, where 22 nucleotides were complimentary to the template DNA and the remaining 22 nucleotides were complimentary to the flanks of the neomycin, noureseothricin, and hygromycin resistance markers. A Selection-5’ (IBO210) primer was designed with 43 nucleotides. Of those, 36 matched the 3’ terminus of the 5’ flank; the remaining 6 nucleotides corresponded to

the 5’ terminus of the selectable marker. The 5’-Selection (IBO209) primer was also 43 nucleotides in length. The 3’-Selection (IBO502) primer consisted of 42 nucleotides, half of which complimented the 5’ terminus of the 3’ gene flank, with the other half complementary to the flanking regions of the three drug resistance markers listed above. All overlap-producing primers (IBO501, IBO502, IBO209, and IBO210) were also PAGE purified. All eight *AGO2* knockout primer sequences are located in Table 3.

Genomic DNA Extraction

Genomic DNA was extracted from overnight cultures of *C. neoformans* grown in liquid medium. Prior to each genomic preparation, *C. neoformans* was inoculated into a 5 mL liquid culture of yeast peptone dextrose (YPD) broth and grown in a G24 Environmental Incubator/Shaker (New Brunswick Scientific Co., Edison, NJ) at 30°C with shaking for 16 hours. After incubation, the cells were pelleted by centrifugation at 13,000 rpm (17,900 x g) and resuspended in an extraction buffer made up of 50 mM Tris-HCl, pH = 8.0, 20 mM EDTA, and 1% SDS. Cells were lysed using a bead-beater (Biospec Products, Bartlesville, OK). Breakage was optimized by drawing out 3-5 µL aliquots of cell suspension and viewing the cells under a LEICA DME light microscope (LEICA Microsystems, Buffalo, NY) at 1000X magnification. Bead-beating was continued until 50-80% breakage was observed. After beating, the cells were incubated at 70°C for ten minutes and then vortexed well. 200 µL of 5 M potassium acetate was added, as well as 150 µL of 5 M NaCl, and the cells were incubated on ice for twenty minutes. Upon completion of the ice incubation, the cells were spun at top speed in a microfuge (14,000 rpm) for twenty minutes and the resulting supernatant was transferred to a clean microfuge tube. 450 µL of chloroform was added to the supernatant and this

solution was centrifuged for an additional ten minutes. The aqueous phase was removed to a clean tube and 200 µL of 30% PEG-8000 was added. The mixture was incubated on ice for ten minutes. After the final incubation, the mixture was centrifuged for ten minutes at top speed in a micro-centrifuge and the supernatant was removed from the resulting pellet. The pellet was resuspended in 50 µL of sterile water and placed at 4°C for short-term storage. No genomic DNA preparations were quantified.

Overlap PCR Protocol

For OL-PCR, two separate rounds of PCR were performed. The first round consisted of the PCR amplification of three separate fragments: the 5' and 3' 1.2 kb flanks of the genes to be deleted, as well as a selectable marker. The selectable marker within the *AGO1* deletion construct was a nourseothricin (NAT) resistance gene that was amplified from the GMC200 plasmid (a gift from Jenny Lodge). The *AGO2* deletion construct contained the hygromycin (HYG) resistance gene as the selectable marker which was amplified from the pTEL-HYG plasmid (a gift from Jenny Lodge).

The first round of PCR was performed with TaKaRa Taq (TaKaRa Bio Inc., Kyoto, Japan). The TaKaRa Taq polymerase has proofreading activity and does not add 3' adenine bases to the amplicons, as most non-proofreading Taq polymerases do. The typical PCR reaction was run using the following master mix recipe: 1 µL (non-quantified) template DNA, 1 µL each of the forward and reverse primer, 5 µL of TaKaRa 10X PCR Buffer (100 mM Tris-HCl (pH=8.3), 500 mM KCl, 15 mM $MgCl_2$, 10% glycerol), 1 µL 10 mM dNTPs, 0.5 µL TaKaRa Taq polymerase (equivalent of 2.5 enzyme activity units), and 40.5 µL sterile water. This 50 µL master mix was divided

into two 25 µL reactions; one contained a test, and the other served as a negative control. Primer concentrations used in the first round of PCRs are shown in Table 4.

Conditions for the first round of PCR were as follows: an initial denaturation step of 30 seconds at 94°C, followed by 28 cycles of 94° for 30 seconds to denature the DNA strands, 58°C for 45 seconds to facilitate primer annealing, and 72°C for 5 minutes which allowed the polymerase to extend the primer, followed by a final extension step of 72°C for 10 minutes to complete any unfinished extensions. PCRs were analyzed via agarose gel electrophoresis to determine if the product had been obtained.

The second PCR used the three fragments obtained from the first PCR as templates. Before this PCR could be carried out, the initial PCR fragments (the 5'flank, the 3' flank, and marker) were purified away from the primers and residual polymerase and buffer. The QIAGEN QIAquick PCR column purification kit (QIAGEN Sciences, MD) was used to perform the necessary purifications. The purified products were analyzed by DNA gel electrophoresis to determine the amount of each template to use in the second PCR.

The second PCR was performed using primers 2 and 7 (Figures 2a and 2b). For this PCR, the AccuPrime™ Pfx Polymerase (Invitrogen, Carlsbad, CA) was used. The typical PCR reaction was run using the following master mix recipe: 1 µL of each of the three non-quantified template DNAs (the 5' flank, the 3' flank, and the marker), 1 µL each of the forward and reverse primer, 10 µL 10X AccuPrime™ Buffer (200 mM Tris-HCl (pH 8.4), 500 mM KCl, 15 mM MgCl2, 2 mM dGTP, 2 mM dATP, 2 mM dTTP, 2 mM dCTP, thermostable AccuPrime™ protein, 10% glycerol), 0.5 µL AccuPrime™ Pfx Polymerase (equivalent of 2.5 enzyme activity units), and 33.5 µL sterile water, divided

into two 25 µL reactions (one test and one negative control). Where LongAmp Taq Polymerase was employed, the typical recipe was as follows: 1 µL of each non-quantified template DNA (the 5' flank, the 3' flank, and the marker), 1 µL each of the forward and reverse primer, 10 µL 5X LongAmp PCR Buffer (final reaction concentrations: 60 mM Tris-SO_4, 2 mM $MgSO_4$, 3 % Glycerol, 0.06 % NP-40, 0.05 % Tween-20, 20 mM ammonium sulfate, pH=9.0), 1 µL 10 mM dNTPs, 1 µL (equivalent of 2.5 enzyme activity units) of LongAmp Taq Polymerase (New England BioLabs, Ipswich, MA), and 33 µL sterile water. This 50 µL master mix was divided into two 25 µL reactions; one contained the necessary templates, and the other served as a negative control. Also, to successfully amplify the nourseothricin marker, dimethylsulfoxide (DMSO) was added at a final concentration of 5% to reduce secondary structures and decrease the melting temperature of the G-C rich nourseothricin DNA fragment. Concentrations of the primers used in the second round (overlap) PCRs are displayed in Table 5.

Conditions for the second round of PCRs were as follows: an initial denaturation step of 2:30 at 95°C, followed by 28 cycles of 95°C for 30 seconds to denature the DNA strands, 58°C (65°C for *AGO2* primers IBO205 and IBO207) for 45 seconds to facilitate primer annealing, and 68°C for 7 minutes which allowed the polymerase to extend the primer, followed by a final extension step of 68°C for 15 minutes to complete any unfinished extensions. All OL-PCRs were done in a PTC-100 Thermal Controller (GMI Inc., Ramsey, MN).

Overlap PCR Optimization

To achieve proper amplification, reactions including DMSO, betaine, and bovine serum albumin (BSA) were attempted. For PCRs containing betaine, the denaturation temperature was decreased to 93°C. Also, to determine the proper annealing temperatures for the primers used, gradient PCR was performed using an Eppendorf MasterCycler Gradient Thermal Cylcer (Eppendorf North America, Westbury, NY). The annealing temperature of 55°C was set with a gradient of ±10°C to determine the most efficient temperature for decreasing non-specific binding of the primers. All OL-PCR products were gel purified using the QIAspin Gel Extraction Kit (QIAgen Sciences, MD) and stored at -20°C.

Molecular Cloning Protocol

Molecular cloning was first described by Chang et al. in 1973, and can be defined as the isolation of a DNA fragment which has been linked to an independently replicating plasmid (molecular vector) within a bacterial cell. As the plasmid replicates, the DNA fragment which is inserted into that plasmid is also replicated by the bacterial host, thus creating cloned copies of the inserted DNA (Hershfield et al., 1974)

The gene region with approximately 1 kb flanks was PCR amplified and cloned into a TA vector in order to create the complementation construct. The typical master mix recipe for these PCRs was as follows: 1 µL template DNA (fresh, non-quantified cryptococcal genomic DNA), 1 µL each of the forward and reverse primer, 10 µL 5X GoTaq PCR Buffer (proprietary make-up; pH=8.5, 7.5 mM $MgCl_2$), 1 µL 10 mM dNTPs, 0.5 µL GoTaq Polymerase (Promega, Madison, WI), and 35.5 µL sterile water. The resulting 50 µL master mix was divided into two 25 µL reactions; one was a test, the

other, a negative control. Parameters for these PCRs consisted of an initial denaturation step of 30 seconds at 94°C, followed by 28 cycles of 94° for 30 seconds to denature the DNA strands, 58°C for 45 seconds to facilitate primer annealing, and 72°C for 7 minutes which allowed the polymerase to extend the primer, followed by a final extension step of 72°C for 15 minutes. Amplicons were generated via GoTaq polymerase (Promega, Madison, WI) such that the PCR products would contain 3'-adenine nucleoside overhangs to allow for ligation of the PCR product into the TA vector that contains thymine overhangs.

Upon successful amplification of the genes, TA-cloning was performed as a means of inserting the gene into a vector that could be subjected to further molecular manipulations. Two separate TA-cloning kits were used for this study; the pCR2.1 TOPO Cloning Vector from Invitrogen (Carlsbad, CA) and the pDrive Vector from QIAgen (QIAgen Sciences, MD). Both vectors contain multiple cloning sites within the *LACZ* gene, and a bacterial origin of replication so that the plasmid can be replicated, as well as kanamycin and ampicillin resistance genes. Schematics of these vectors are illustrated in Figures 3 and 4, respectively.

Ligations were carried out per the manufacturer's recommendations. Insertions into the pDrive Vector were ligated by adding 1 µL of the 50 ng/µL vector, 5 µL of2X Ligation Master Mix, 2 µL of fresh, non-quantified, column purified PCR product, and brought to a final volume of 10 µL with sterile water. This ligation reaction was incubated at 16°C for 2.5 hours as the manufacturer recommended. Insertions into the pCR2.1 TOPO vector were performed by adding 1µL of the 10 ng/µL TOPO Vector, 2 µL of fresh, non-quantified un-purified PCR product, 1 µL of the supplied salt solution

(1.2 M NaCl and 0.06 M $MgCl_2$), and brought to a final volume of 6 µL with sterile water. These ligations were incubated at room temperature for 30 minutes.

Bacterial Cell Transformation

Ligation reactions were transformed into chemically competent DH5α cells (Fisher Scientific, Pittsburgh, PA). 40 µL of cells were thawed on ice and 2 µL of the ligation was added. The ligation/cell mixture was incubated on ice for 30 minutes. Cells were then heat-shocked at 37°C for 45 seconds, and 300 µL of Luria Bertani (LB) broth was added. The newly transformed cells were incubated at 37°C with shaking for one hour in a G24 Environmental Incubator/Shaker (New Brunswick Scientific Co., Edison, NJ) and then plated onto LB plates containing ampicillin (AMP) and X-gal. Ampicillin killed the cells that did not receive the plasmid. X-gal allowed for the screening of plasmids that had ligated without the incorporation of the PCR product because the LAC operon was functional in these bacteria, and the colonies appeared blue. Colonies that had the insert appeared white and were harvested for colony PCR.

Colony PCR Protocol

Colony PCR is a PCR-based technique that tests for the presence of an inserted sequence from a plasmid without costly and time-consuming plasmid DNA preparations from numerous candidate bacterial colonies obtained from a single transformation. White colonies from the transformed ligations were selected and master plated on to an LB+AMP agar plate using a sterile toothpick. The plate was incubated at 37°C for ~16 hours to allow the cells to grow. Immediately after streaking the colony onto the master plate, the toothpick was swirled in 5 µL sterile water that had been aliquoted into a 0.5 mL PCR tube. The DNA within the cells that were shed from the toothpick into the

water served as template for the reaction. Taq polymerase, dNTPs, 5X GoTaq PCR Buffer, and primers (to amplify the inserted gene) were added in the proportions described above. PCR was facilitated by an initial denaturing step of 2 minutes at 94°C (to break open the cells) and followed the standard PCR protocol described above. Products were analyzed via DNA gel electrophoresis for the presence of an amplicon. All colony PCR reactions were performed using an Eppendorf MasterCycler Personal (Eppendorf North America, Westbury, NY). Primers used to amplify the different genes in colony PCR reactions are displayed in Table 6. These primers were designed to amplify an approximate 600 bp section of the gene in order to perform PCR with minimal extension times.

Plasmid Preparation

In samples where an amplicon was present, cells were harvested from the master plate and grown in LB+AMP liquid broth with shaking for ~16 hours prior to plasmid preparation. After overnight growth, the bacterial cells were harvested from the LB+AMP broth by centrifugation and plasmid DNA was extracted from the cells using the QIAspin MiniPrep Kit (QIAgen Sciences, MD). Plasmid DNA was eluted in 35 µL of sterile water and stored at 4°C. To confirm the presence of an inserted DNA fragment, subsequent enzymatic plasmid digestions were performed. The plasmid DNA was also diluted 1:10 with sterile water and used as a template for PCR as described above.

Determining Restriction Sites for Plasmid Digestion

SerialCloner v. 1.3, a DNA alignment software available from http://serialbasics.free.fr/Serial_Cloner.html, was used in this study to determine which enzymes could be employed to remove the open reading frame (ORF) of the inserted

genes (for knockout constructs), as well as which enzymes would cut only once and allow for the formation of the complementation constructs. SerialCloner was also used as an *in silica* experimentation suite to perform PCR and restriction digests prior to running actual experiments *in vitro*. Both the TOPO (Figure 3) and the pDrive (Figure 4) vectors contain a single EcoRI restriction site on each side of the insert. Thus, plasmids thought to contain an insert were digested with EcoRI and the presence of an insert was confirmed or rejected based on the banding pattern.

Restriction Endonuclease Reactions

Vectors known to contain the inserted gene were linearized in order to create complementation constructs for the corresponding deletion constructs. Pilot experiments were performed to confirm that the enzyme would indeed cut as it should given the correct conditions as supplied by the manufacturer (New England BioLabs, Ipswich, MA). For these small-scale experiments, 3 µL of fresh prepared, non-quantified, TOPO-*AGO1* and pDrive-*DICER2* plasmid DNA were digested separately by 1µL (equal to 100 enzyme activity units) of the MfeI enzyme (New England BioLabs, Ipswich, MA), 1.5 µL of the supplied 10X NEB 4 reaction buffer (50 mM potassium acetate, 20 mM Tris-acetate, 10 mM magnesium acetate, 1 mM dithiothreitol, pH 7.9), 1.5 µL of 10X BSA (where required), and water was added to a final volume of 15 µl. The digests were incubated in a 37°C water bath for approximately 2-3 hours to carry out the digestion. Upon completion of the incubation period, the digested plasmid was analyzed by DNA gel electrophoresis to determine if the digestion had proceeded as expected.

After pilot experiments confirmed the enzyme's ability to produce the correct banding pattern, full digests were done as follows: 20 µL plasmid DNA, 3 µL of MfeI

(equal to 300 enzyme activity units), 6 µL supplied 10X NEB 4 reaction buffer, 6 µL 10X BSA (where required), and water to a final volume of 60 µL. Digests were incubated for four hours in a 37°C water bath.

Plasmid Processing

Immediately following digestion of the plasmids, the digestion reactions were inactivated by heat incubation per the manufacturer's instructions (where applicable). If particular enzymes were not able to be heat inactivated, column purification was performed using the QIAquick Column PCR Purification kit (QIAgen Sciences, MD). Once the restriction enzymes had been inactivated or removed from the reaction, the ends of the DNA fragments were blunted by adding 1 µL, or 3 enzyme activity units, of T4 DNA Polymerase (New England BioLabs, Ipswich, MA) and 3 µL of 2 mM dNTPs and incubating at 16°C in the MasterCycler Personal Thermal Cylcer (Eppendorf North America, Westbury, NY) for twenty minutes, followed by a step of 75°C for 10 minutes to heat-inactivate the polymerase. After blunting, all vector fragments (i.e. not inserts) were treated with a calf intestinal alkaline phosphatase (CIP) to remove the 5' phosphates from the vectors and prevent the vector from ligating to itself, decreasing background. CIP treatment was performed by adding 1 µL (10 enzyme activity units) of CIP and incubating in a 37°C water bath for thirty minutes. The vectors and inserts needed from the various digests were retrieved via gel purification with the QIAspin Gel Extraction Kit (QIAgen Sciences, MD).

Ligation Protocol

For the complementations, a neomycin resistance gene was inserted into the 3' flank of the gene such that when introduced, the complementation would re-introduce the

wild type gene (Figure 5). Ligation reactions were setup using a TOPO+*AGO1* plasmid that had been linearized with the MfeI restriction enzyme and the G418 selectable marker insert obtained from a HindIII digestion of the TDB546 plasmid. Table 7 describes the typical makeup of a ligation reaction. Reactions were facilitated by an overnight, room temperature incubation period that allowed T4 DNA Ligase (New England BioLabs, Ipswich, MA) to join the insert and the vector, resulting in a complementation construct of *AGO1*. Successful ligations were digested with restriction enzymes to determine the orientation of the insert within the plasmid. Constructs were transformed into DH5α *E. coli* as described previously and frozen down to -80°C for long-term storage.

Freezing Stocks of Plasmid-Containing *Escherichia coli*

Once cultures of *E. coli* cells were confirmed to contain any plasmid of interest (TOPO+*AGO1*, TOPO+*AGO2*, complemented genes, deleted genes, etc.) by colony PCR and plasmid digestion, cells were grown overnight in 5 mL of LB+AMP broth and prepared for storage at -80°C. Tubes were clearly labeled with the strain name, the plasmid name, as well as the selectable markers contained in the plasmid, and this information was also entered into a database shown in Table 9. 500 µL of fresh cell culture was mixed with 500 µL of 50% glycerol in sterile water. The cells were vortexed well and placed on dry ice briefly. The cells were placed at -80°C in a Revco Ultima II Ultralow Freezer (Thermo Fisher Scientific, Waltham, MA) for long term storage of the cells containing the plasmid DNA.

RESULTS

The overall goal of this work was to create strains of *Cryptococcus neoformans* lacking the *AGO1*, *AGO2*, *DICER1* and *DICER2* genes; as well as to create complementation strains of these deletions. Gene deletion was attempted employing two methods: overlap PCR and molecular cloning. Overlap PCR proved to be the more efficient of these two, allowing the creation of deletion constructs for both *AGO1* and *AGO2*. Molecular cloning was used, however, in an attempt to create complementation constructs for the *AGO1* deletion. The *AGO2* deletion will be addressed later. For unknown reasons, the *DICER1* gene was not amplified via PCR. Therefore, the *DICER2* gene was abandoned, and the focus shifted to the *AGO1* and *AGO2* genes.

Overlap PCR

Once sequence information is obtained, primers are designed to amplify the 5' flank of the gene. The forward primer consists of 20-25 complimentary bases to the 5'terminus of the 5' flank; however, the reverse primer is designed such that 20-25 bases match the template DNA and an additional 15-20 base portion which matches the 5' terminus of the marker to be used to delete the gene.

The marker region is amplified using a forward primer of approximately 40 bases, 20 which match the marker and 20 which match the 3' terminus of the 5' flank. The reverse primer used to amplify the marker contains 40 bases, 20 which match the marker and 20 which match the 5' terminus of the 3' flank.

The 3’ flank of the gene is amplified using a forward primer that contains a stretch of 20 nucleotides complimentary to the 3’ terminus of the marker, as well as 20 nucleotides which compliment the 5’ terminus of the 3’ flank of the gene. The reverse primer used to amplify the 3’ flank compliments the 3’ terminus of the 3’ flank (Figure 2a).

The three separate amplicons achieved via the first round of PCR are then used in a second round. Figure 2b illustrates the three fragments being fused by a PCR reaction using two inside primers. The overlapping regions of the fragments allow the polymerase to amplify the 5’ flank, fusing it to the marker, and then fusing that piece to the 3’ flank, creating a fusion product consisting of the gene flanks with a marker in the middle.

The three preliminary PCRs were obtained from fresh genomic DNA preparations of *C. neoformans* strain KN433α (serotype D) and purified using the QIAgen PCR purification kit columns (QIAgen Sciences, MD) (Figure 6a). These amplicons were generated using the TaKaRa Taq Polymerase (TaKaRa BioSciences, Kyoto, Japan). Overlap PCR was attempted and the results showed slight amplification of some intermediate fragments, but not the entire overlap PCR we had hoped to generate (Figure 6b).

To address the issue of template concentration, the OL-PCRs for the *AGO1* and *AGO2* deletion constructs were set up using various dilutions of template. Each PCR was run with a 1:10 as well as a 1:20 dilution of the original genomic preparation (dilutions were created by addition of sterile water). However, these PCRs gave no fragment of the expected size, and often showed the amplification of non-specific fragments.

The non-specific banding pattern could be the result of the annealing of primers to non-specific regions of the genome. This can be addressed by varying the annealing temperature of the reaction to determine the best annealing temperature for a primer pair. Annealing temperature was optimized for the *AGO2* amplicon. Figure 7 clearly shows a decrease in non-specific banding as the annealing temperature is increased toward an optimum. Using this technique, the optimal annealing temperature for primers IBO205 and IBO207 amplifying the *AGO2* gene was determined to be approximately 65°C. However, although this reduced the non-specific bands, the amplification of the specific overlap PCR fragment did not improve with annealing optimization.

Since template concentration changes and varying annealing temperatures of primers did not give the desired result, additional adjuncts that are known to improve PCR efficiency were tried in the overlap PCR procedure. These adjuncts included the small molecules dimethyl sulfoxide (DMSO), dithiothreitol (DTT) and N,N,N-trimethylglycine (betaine). PCRs were first tested with a final concentration of 1.5 M betaine, but these reactions showed no improvement. Betaine should decrease non-specific binding and aid in DNA melting, allowing for more efficient amplification. However these data show that, for the primer pairs that were used, betaine actually inhibited PCR, even at the recommended concentration (data not shown). Next, DMSO was added at a final concentration of 5%. Like betaine, DMSO aids in DNA melting (especially those templates which have a high G-C content) while also decreasing secondary structure formation within single-stranded DNAs. Following the work of Rasler et al. (2006), I also tried to use these adjuncts in combination to determine if that would allow specific fragments to be amplified with greater efficiency. Overlap PCR

was repeated using the same amplicons shown in Figure 6a with the addition of a combinatorial enhancement solution (CES) (Rasler et al., 2006). The CES was prepared as a 5X stock solution, and the recipe can be found in Appendix I. Because of the betaine constituent of the CES, the denaturation temperature of the PCR was lowered to 93°C. This PCR showed some improvement over the original gel; however, the overlapping fragment was not achieved (data not shown).

Heat-stable DNA polymerases are an integral part of the PCR process. These enzymes allow for the selection and addition of the complementary base to the growing strand of DNA during the PCR reaction. However a plethora of heat-stable DNA polymerases are found in today's market. These enzymes fall within two major categories: non-proofreading and proofreading DNA polymerases. The non-proofreading polymerases, although usually very robust, have less fidelity and are liable to make more mistakes while adding new bases (Ignatov and Kramarov, 2009), whereas the proofreading polymerases, although more accurate, are often less robust enzymes. I used a number of these polymerases in order to determine which type of enzyme functions best for our purposes. The non-proofreading polymerase GoTaq (Progmega, Madison, WI), and the proofreading polymerases TaKaRa Taq (TaKaRa Bio, Japan), and KTLA Taq (Wayne Barnes, Washington University) were not able to produce an overlap, although each one was capable of producing the preliminary PCRs and the entire gene region. Since the proofreading polymerases are known to be less robust, cocktails of Taq polymerases were also formulated with the idea that if proofreading polymerases can initiate the production of the overlap PCR fragments, it might be possible for the more active non-proofreading enzymes to take over and increase the yield of the fragment. In a

particular PCR, 0.25 µL (1.25 units of enzyme activity) of a robust non-proofreading Taq and 0.25 µL (1.25 units of enzyme activity) of a proofreading Taq were both added to the master mix, but this did not give the expected results.

The overlap PCR was not obtained using any of the above parameters (data not shown). Template concentration, primer annealing temperature, and the type of polymerase used were all systematically ruled out as being the reason for the failure of overlap fragment amplification. I have also shown that, with the primer pairs in use, addition of various adjuncts did not improve the reaction result. It was hypothesized that perhaps these amplicons were too cumbersome for the PCR protocol being used. To test this, the entire gene regions of *AGO1, AGO2, DICER1,* and *DICER2* were amplified using the outside primers for each (Tables 2 and 3). The resulting bands are shown in Figure 8. GoTaq polymerase (Promega, Madison, WI) successfully amplified *AGO1*, *AGO2,* and *DICER2*. This showed that the length of the fragment being produced was not the limiting condition for the reaction.

The results of a PCR using PAGE-purified primers to generate the preliminary PCRs are shown in Figure 9. The resulting preliminary PCRs were used in an overlap PCR. The gel is shown in Figure 10. To increase the yield of these PCRs, another Taq polymerase, AccuPrime^{TM} Pfx (Invitrogen, Carlsbad, CA), was acquired and used to generate the preliminary PCRs (Figure 11) and to perform an overlap PCR (Figure 12). The *AGO1* deletion construct was successfully generated, as seen in Figure 12. To show this, Figure 13 represents a schematic representation of the *AGO1* OL-PCR product. *AGO2,* although present, showed a low yield. To further increase the yield of an *AGO2* overlap deletion fragment, a few optimization experiments were conducted, including

adding betaine and DMSO separately, as well as increasing the annealing time from 30 seconds to 45 seconds (data not shown). Because no increase in yield was obtained, five individual PCRs were run without adjuncts (Figure 14). These PCRs were pooled into one tube and the overlap fragment was gel purified (Figure 15). A schematic representation of the *AGO2* deletion construct is illustrated in Figure 16.

Molecular Cloning

Concurrently with overlap PCR, molecular cloning techniques were engaged. The *AGO1, AGO2,* and *DICER2* genes were successfully amplified via PCR (Figure 8). These amplicons were then successfully ligated into a vector. Both the *AGO1* and *AGO2* genes were successfully ligated into the pCR2.1 TOPO cloning vector from Invitrogen Inc. (Carlsbad, CA), while *DICER2* was ligated into the pDrive vector from QIAgen Sciences (QIAgen Sciences, MD). These ligations were tested via colony PCR and then confirmed through plasmid DNA preparation and digestion with EcoRI (New England Bio Labs, Ipswich, MA) (data not shown). The TOPO cloning vector had greater ligation efficiency than the pDrive vector. Of the 45 white colonies screened for a pDrive plasmid, only one showed an insert, the single pDrive-*DICER2* plasmid. The two TOPO-*AGO2* plasmids were identified after screening only 17 white colonies, and the lone TOPO-*AGO1* plasmid was confirmed with only 16 white colony screenings. However, for the purposes of this study, the pDrive-*DICER2* plasmid was not investigated due to the co-required dicer gene (*DICER1*) which was unable to be amplified.

The linear plasmids are shown on an agarose gel in Figure 17. The sequences of these plasmids were checked for possible restriction sites for the insertion of the complementation marker. The TOPO-*AGO1* plasmid was found to have a single cut site

for the restriction enzyme MfeI (New England Biolabs, Ipswich, MA) which was located 127 bases from the *AGO1* gene stop codon. Ligating the linear TOPO-*AGO1* plasmid (digested with MfeI) with the neomycin resistance marker (obtained from the TDB546 plasmid via digestion with HindIII) created an *AGO1* complementation construct (Figure 18). These constructs were tested by colony PCR and gave several results. The first eight colonies showed a positive, approximately 800 base pair band that lined up with the TDB546 positive control plasmid PCR (Figure 19). However, there was also an unexpected band of approximately 2,000 base pairs with these PCRs (Figure 19). To address the unexpected 2,000 base pair band, a separate PCR was run using the TDB546 neomcycin –containing plasmid and the IBB103 plasmid, which also contained the neomycin cassette (Figure 20). The results from this PCR (Figure 20) were consistent with the results of the earlier PCR (Figure 19). Descriptions of these colonies can be found in the complete database of frozen *E. coli* (Table 9).

Two of the eight colonies that contained the *AGO1*(Neomycin) plasmid were subjected to further testing in order to determine the orientation of the insert. When ligations are performed, the insert can orient in two separate ways. The insert can be incorporated into the plasmid in the forward orientation (i.e., the same orientation it was within the source plasmid) or in the reverse orientation. A schematic of the differences in orientation can be found in Figure 21.

The DNA shown in Figure 22 is the result of a EcoRI digestion of the *AGO1*(Neomycin)#1 and *AGO1*(Neomycin)#2 vectors. The complementation construct *AGO1*(Neomycin)#1 was found to be in the correct forward orientation, whereas the complementation construct *AGO1*(Neomycin)#2 was found to be in the reverse

orientation. Although this does not affect the validity of any complementation construct, it is an important quality control step, because knowing the composition of the nascent construct is of paramount importance.

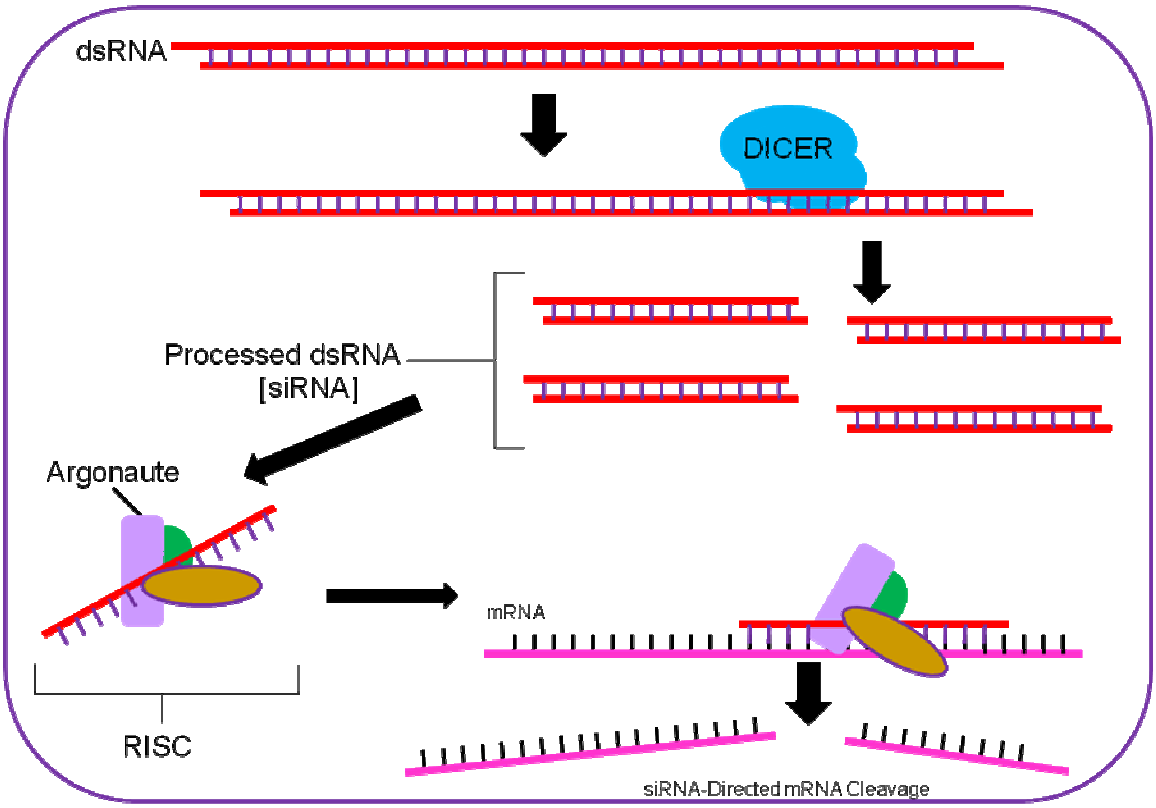

Figure 1. The RNAi pathway. dsRNA in the cytoplasm is digested into siRNAs by the Dicer protein. The siRNAs are then unzipped and loaded into the RISC in an ATP-dependent manner. The RISC, armed with the ss-siRNA, finds the complimentary mRNA strand and binds it. Upon binding with the mRNA, the Argonaute protein (rectangle) cleaves the mRNA at the 3'end, preventing translation into protein.

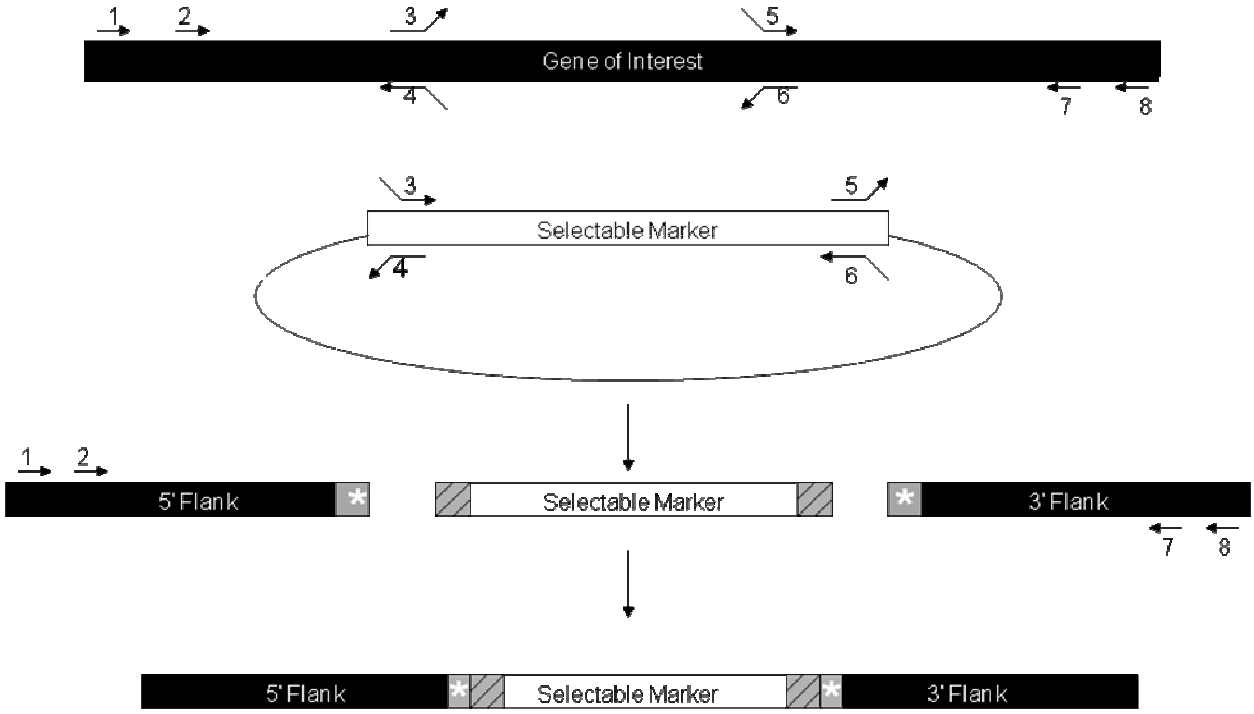

Figure 2a. Overlap PCR Amplification. The procedure is performed as follows: primers 1 and 4 are used to amplify the 5' flank of the gene; primers 8 and 5 are used to amplify the 3' flank of the gene; primers 3 and 6 are used to amplify some marker. The products of these PCRs are then used as templates in a reaction containing primers 2, and 7, which yield the final piece of DNA consisting of the 5' flank fused to a selectable marker and the 3' flank of the gene. This figure was adapted from Davidson, et al., 2002.

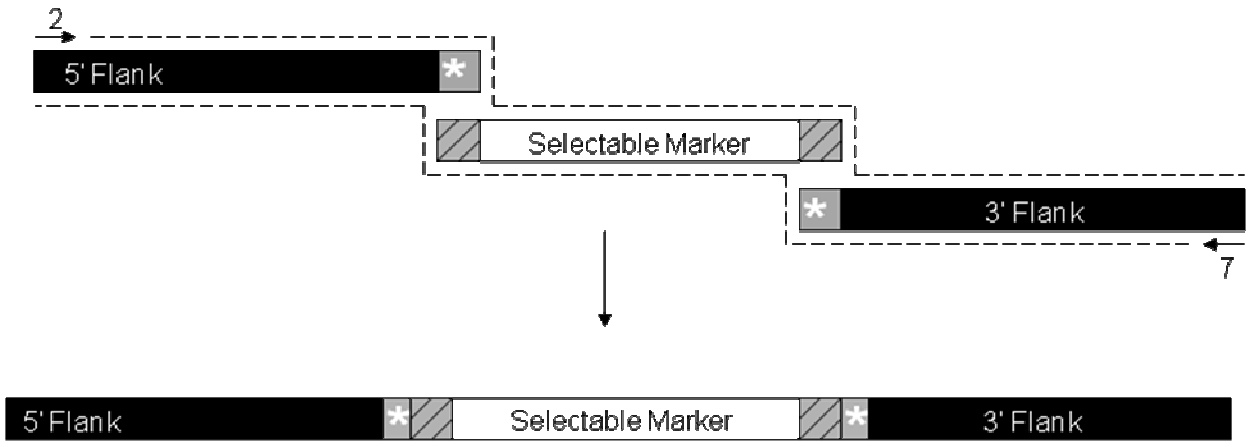

Figure 2b. OL-PCR of three different PCR products. Primers 2 and 7 amplify the 5' flank, the marker, and the 3' flank of the gene to be disrupted. Here, the overlapping of the flanks and the marker allow for amplification throughout, resulting in one whole PCR product (deletion construct) containing the 5' gene region and the 3' gene region with a marker inserted where the mid-section of the gene would be.

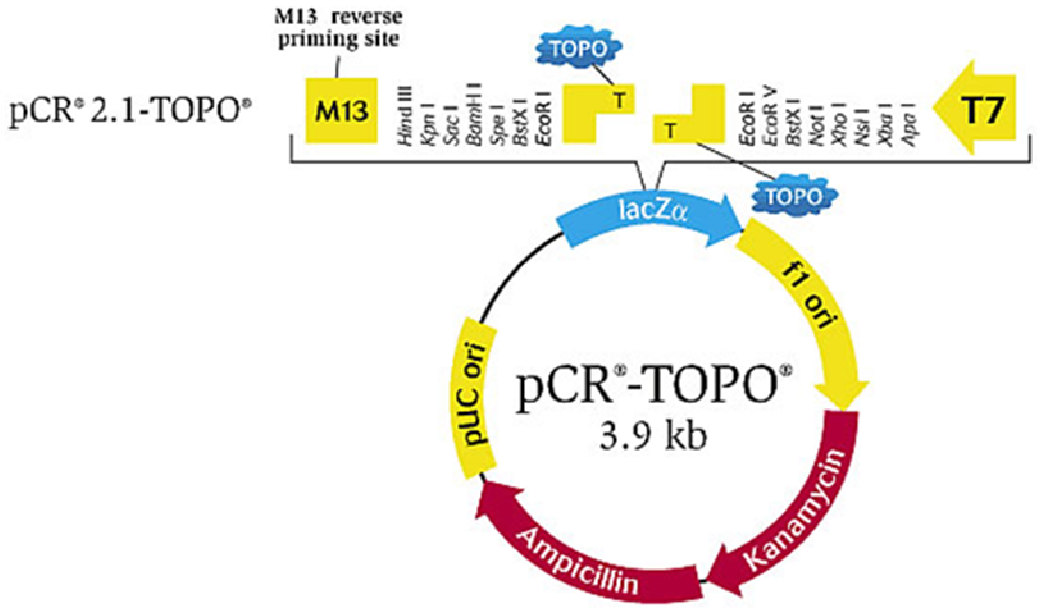

Figure 3. Graphic representation of the pCR2.1 TOPO vector from Invitrogen (Invitrogen, Carlsbad, CA). This vector contains an associated topoisomerase and yields a high cloning efficiency of PCR products. It contains ampicillin and kanamycin bacterial selection, a pUC origin, an f1 origin, and the multiple cloning site lies within the lacZα gene to allow for blue/white screening. The plasmid also contains an M13 cloning site and a T7 promoter. The figure above was downloaded from the Invitrogen website, www.invitrogen.com.

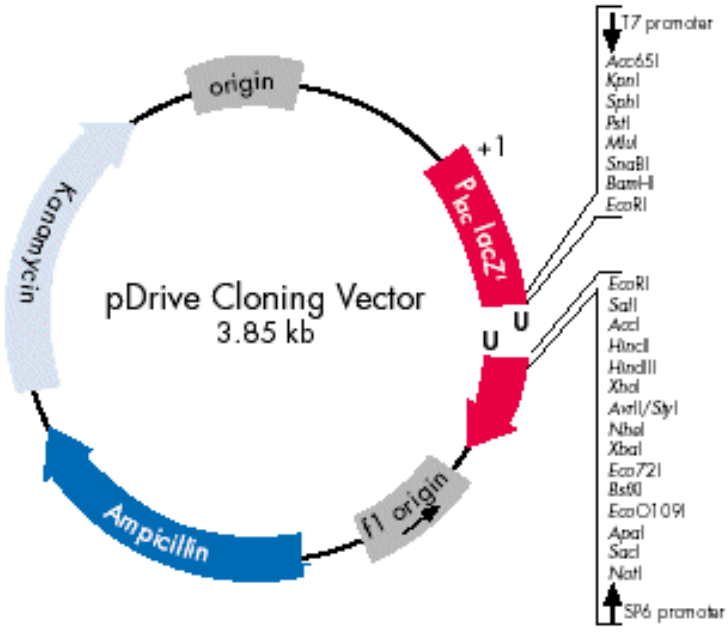

Figure 4. Graphic representation of the pDrive cloning vector from QIAgen (QIAgen Sciences, MD). The vector contains both T7 and SP6 promoter which flank the cloning site. The cloning site is located inside the lacZα gene to facilitate blue/white screening. This plasmid also contains both ampicillin and kanamycin bacterial selection, and possesses an f1 origin. The figure above was downloaded from the QIAgen website, www.qiagen.com.

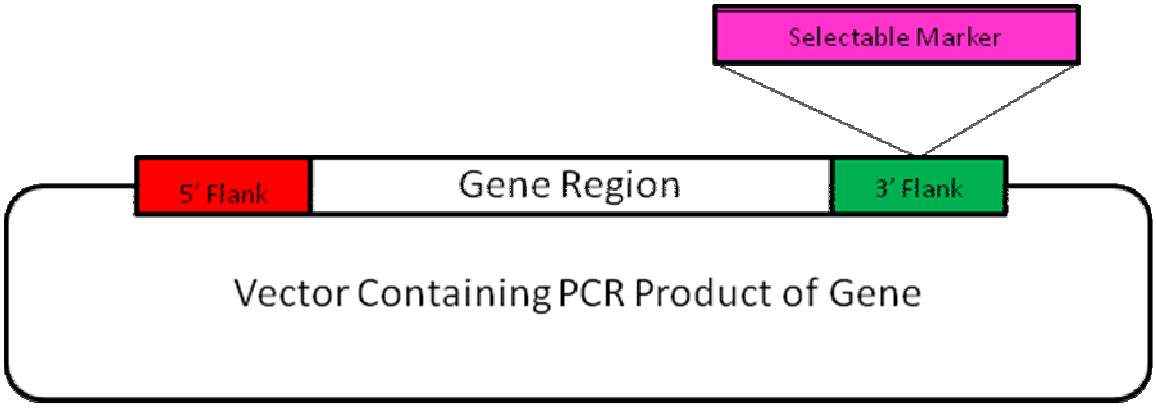

Figure 5. Creation of a complementation construct to re-introduce the wild type gene. The selectable marker is placed inside the 3' flank approximately 300bp downstream of the stop codon, leaving the coding region of the gene intact while allowing for selection of colonies that received the resistance marker.

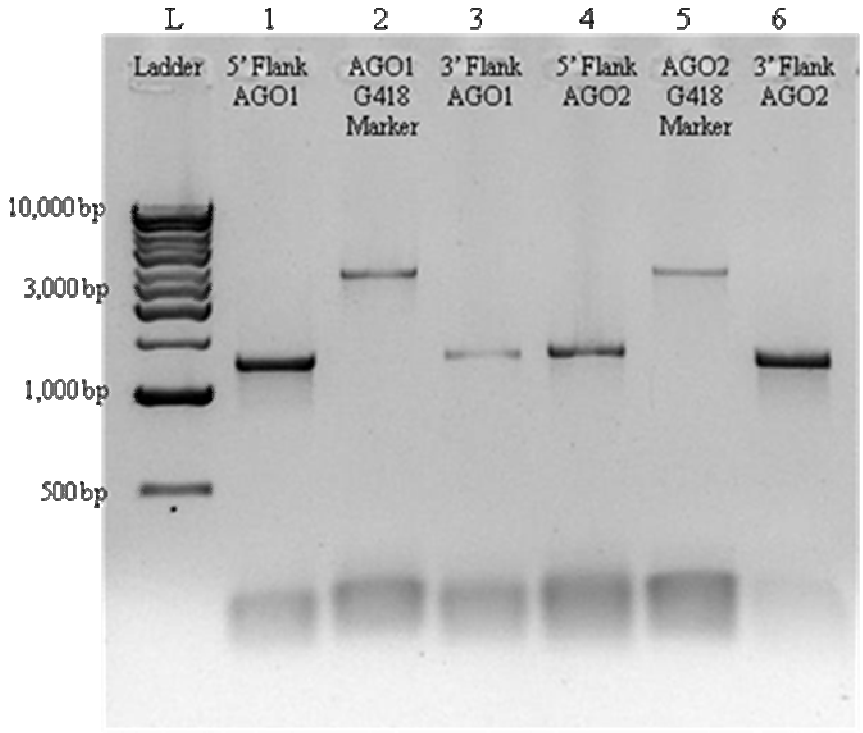

Figure 6a. Agarose gel of purified preliminary amplicons generated from *C. neoformans* genomic DNA that were used in an overlap PCR procedure. Lane 'L' contains the DNA ladder labeled with appropriate base pair sizes. Lanes 1-3 contain the 5' flank, the G418 marker, and the 3' flank of the AGO1 gene, respectively. Lanes 4-6 contain the 5' flank, the G418 marker, and 3' flank of the AGO2 gene, respectively.

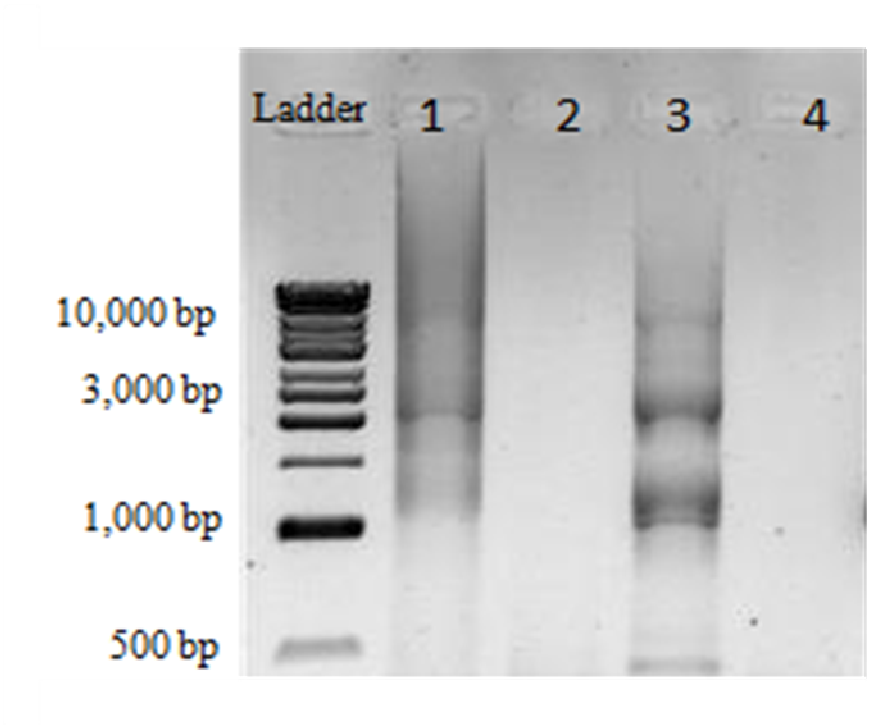

Figure 6b. Agarose gel of overlap PCR using the amplicons from Figure 6a with GoTaq polymerase. The far left 1ane contains a DNA ladder labeled with the appropriate base pair sizes. Lanes 1 and 2 are the test and negative control, respectively, for the *AGO1* overlap. Lanes 3 and 4 are the test and negative control, respectively, for the *AG02* overlap. No overlap was generated in these PCRs. If the overlap had been generated, a band would be expected at approximately 5 kb for both *AGO1* and *AGO2*.

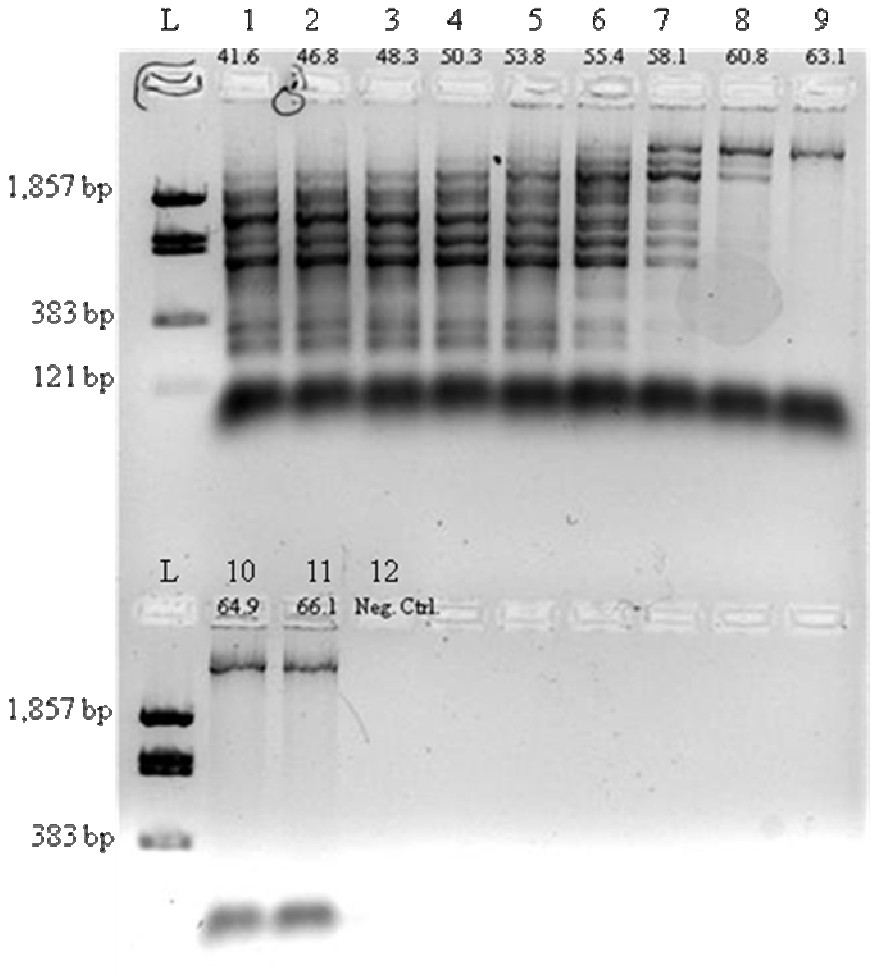

Figure 7. Gradient PCR of the *AGO2* overlap. Annealing temperature was programmed for 54°C±10°C. The two 'L' lanes contain a DNA ladder marked with the appropriate base pair sizes. Each lane is labeled with a number 1-12. The numbers underneath the lane number designate the annealing temperature used in degrees Celsius. All PCRs are the *AGO2* OUT amplicon generated by primers IBO205 and IBO207 (Table 3) with the Go Taq polymerase. Optimal annealing temperature for these primers is approximately 65°C, as seen in lanes 9-12.

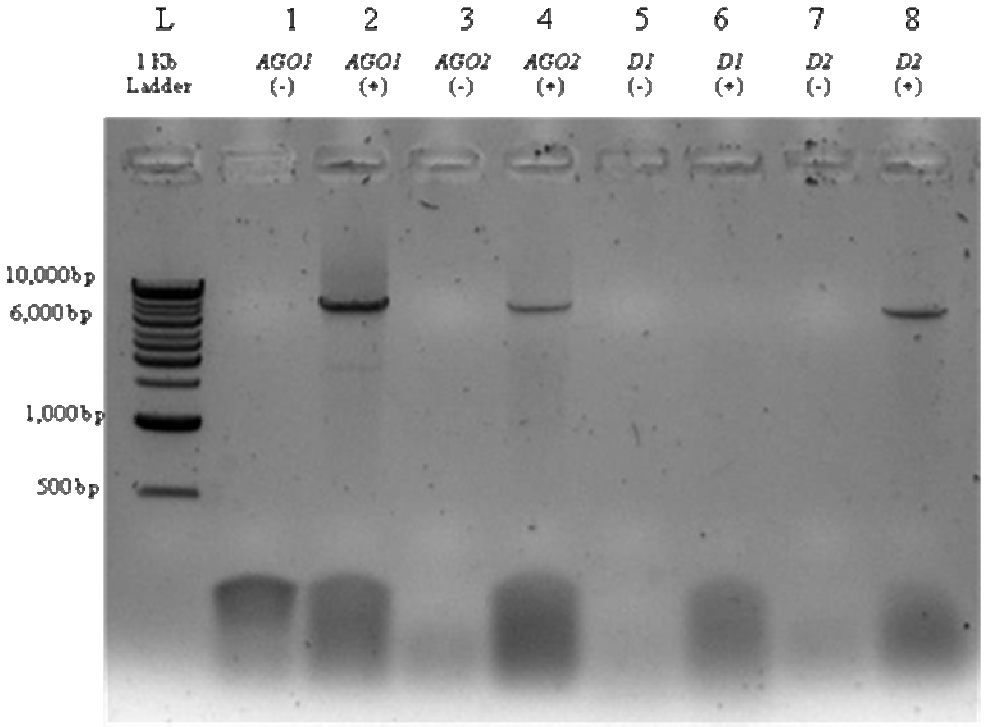

Figure 8. PCR of the entire *AGO1, AGO2, DICER1* & *DICER2* genes with approximately 1Kb flanks. The 'L' lane contains a 1Kb DNA ladder, and band sizes of the different amplicons match those expected (Table 1). Lanes 1 and 2 represent the negative control and the *AGO1* gene, respectively. Lanes 3 and 4 represent the negative control and the *AGO2* gene, respectively. Lanes 5 and 6 represent the negative control and the *DICER1* gene, respectively. Lanes 7 and 8 represent the negative control and *DICER2* genes, respectively.

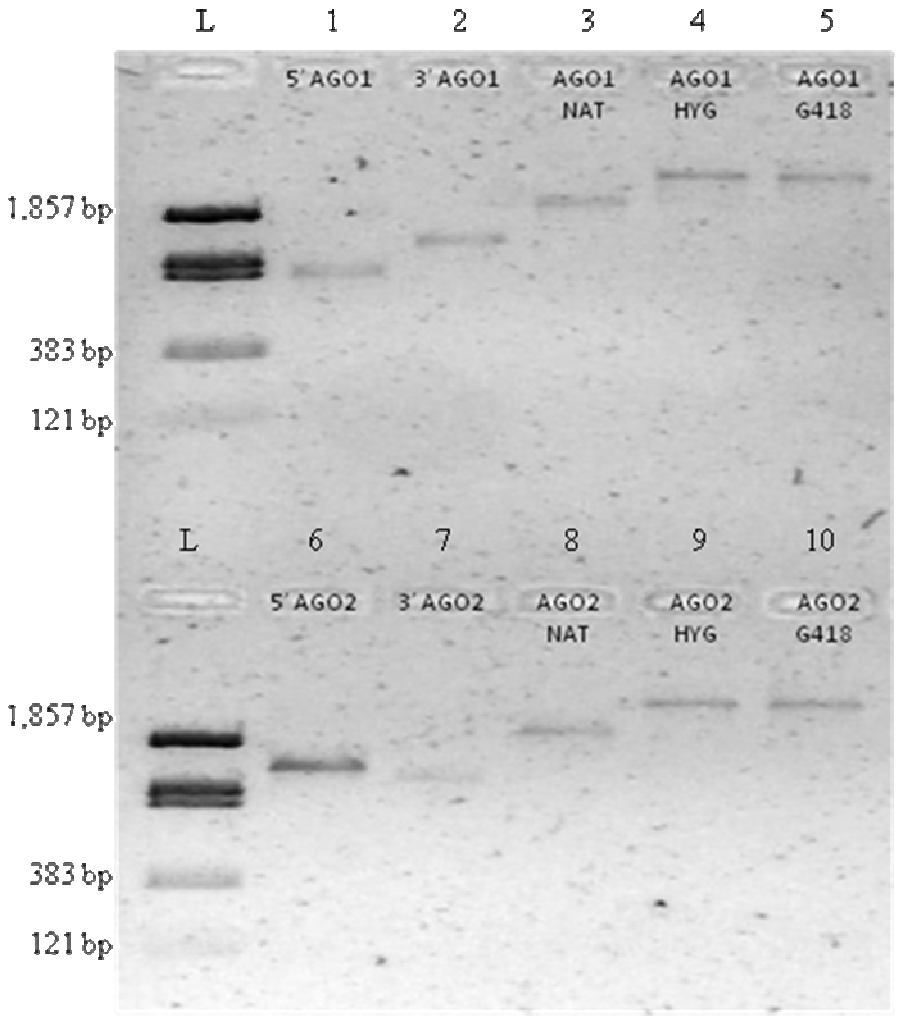

Figure 9. Preliminary PCRs for deletion constructs. These amplicons were generated using PAGE-purified primers (Tables 1 and 2). The left-most lane of each row, the 'L' lane, contains a marker labeled with the appropriate base pair sizes. This gel shows a low yield of the 5' flank of *AGO1* (Lane 1), the 3' flank of *AGO1* (lane 2), and the NAT, HYG, and G418 drug resistance markers containing overlapping regions to the *AGO1* gene (lanes 3, 4, and 5). The bottom row consists of the same amplicons, except these pertain to *AGO2.* Lane 6 holds the 5' flank. Lane 7 holds the 3' flank. Lanes 8, 9, and 10 hold the NAT, HYG, and G418 amplicons for *AGO2*.

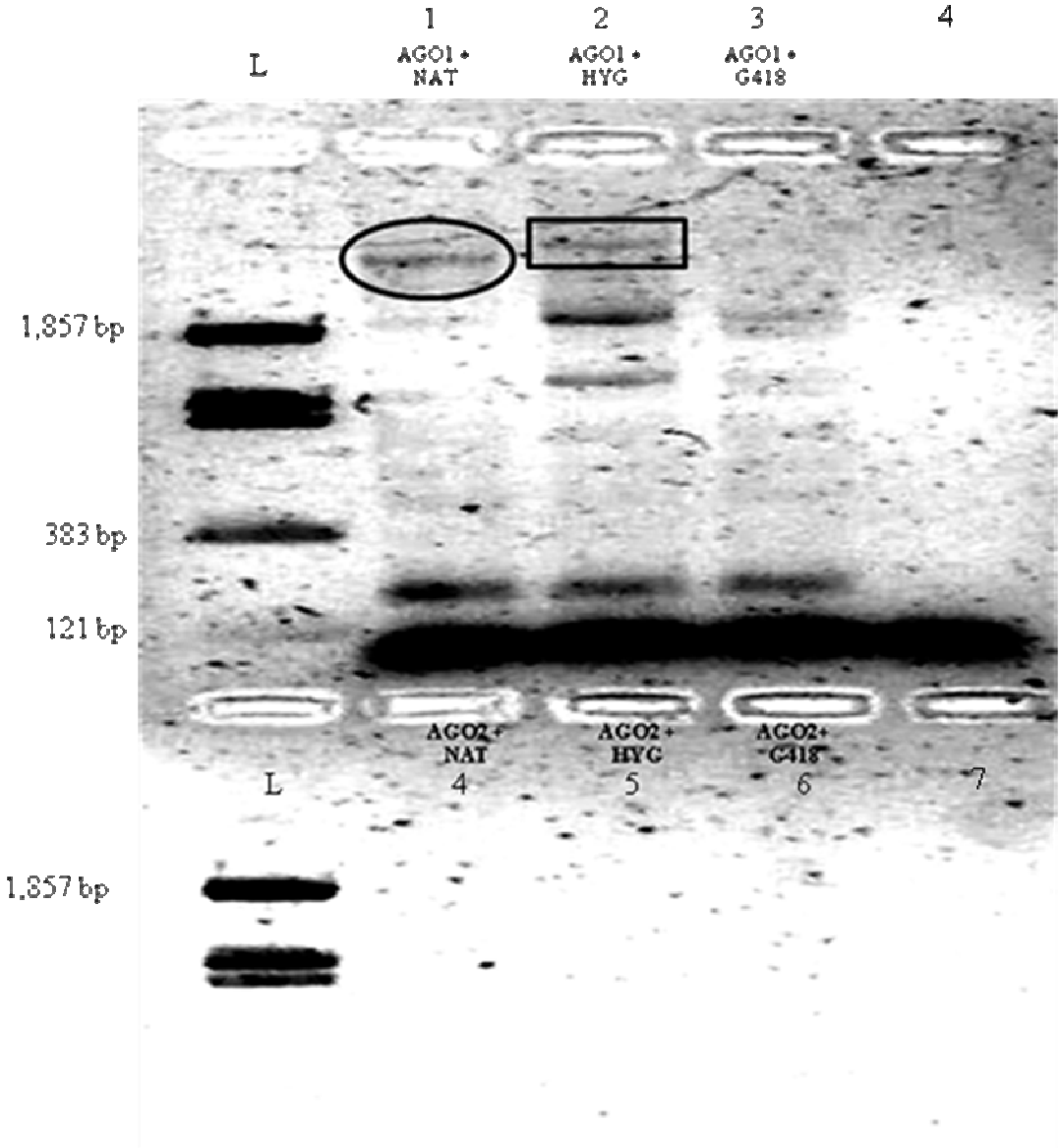

Figure 10. Overlap PCR deletion constructs using fragments shown in Figure 9. The 'L' lanes contain a DNA ladder marked with the appropriate base pair sizes. This agarose gel depicts an amplicon consisting of the 5' and 3' flanks of the *AGO1* gene with a nourseothricin selectable marker replacing the coding region of the gene in lane 1. This amplicon is emphasized by the black oval. The black rectangle shows the 5' and 3' flanks of the *AGO1* gene with a hygromycin selectable marker replacing the coding region of the gene in lane 2. The *AGO1* + G418 overlap shows no result (lane 3). The bottom row of this gel depicts the unsuccessful overlap PCRs of the *AGO2* gene with the nourseothricin, hygromycin, and the G418 neomycin resistance markers (lanes 4-6). Lanes 4 and 7 contain the negative control for these PCRs.

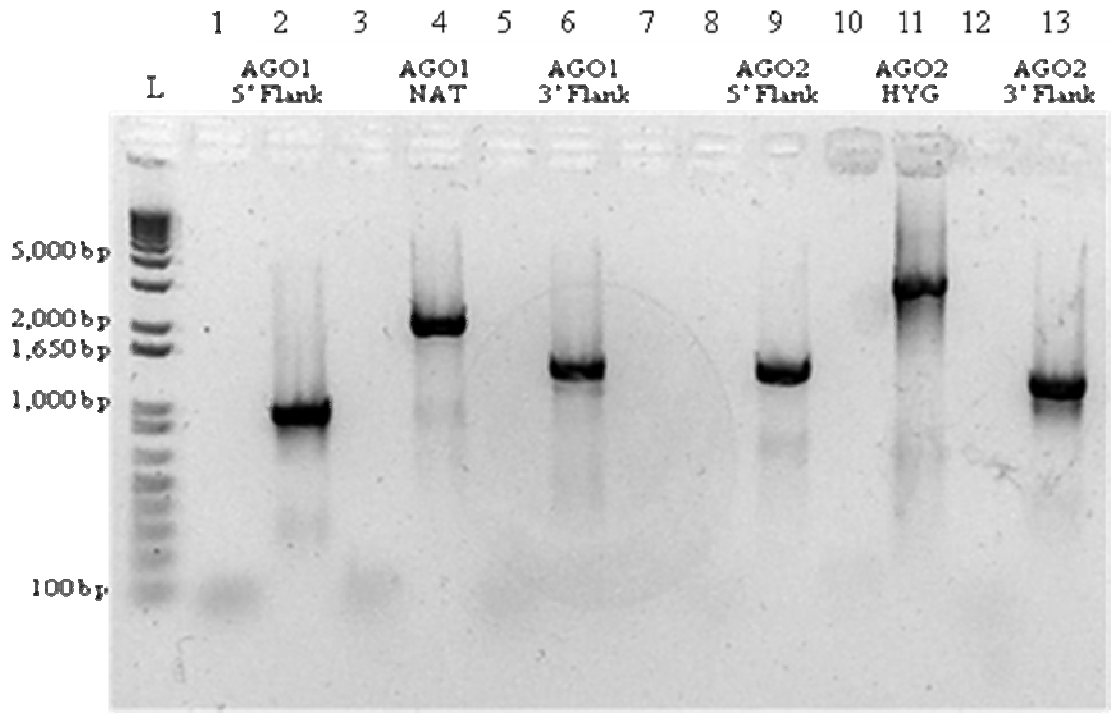

Figure 11. Preliminary PCRs for overlap performed with the AccuPrime™ Pfx Taq Polymerase (Invitrogen Inc., Carlsbad, CA) and PAGE purified primers. The 'L' lane contains a DNA marker labeled with the appropriate base pair sizes. Lane 1 contains the negative control for the 5' flank of *AGO1* and lane 2 contains the test. Lane 3 contains the negative control for the nourseothricin marker with *AGO1* overlaps, and lane 4 shows the test. Lane 5 contains the negative control of the 3' flank of the *AGO1* gene, whereas lane 6 shows the test. Lane 7 was intentionally left blank. The negative control for the 5' flank of *AGO2* is located in lane 8, with the test PCR being in lane 9. Lane 10 contains the negative control for the hygromycin resistance gene with *AGO2* overlaps, where lane 11 contains the test PCR. Lane 12 contains the negative PCR the 3' flank of *AGO2*, where lane 13 contains the test.

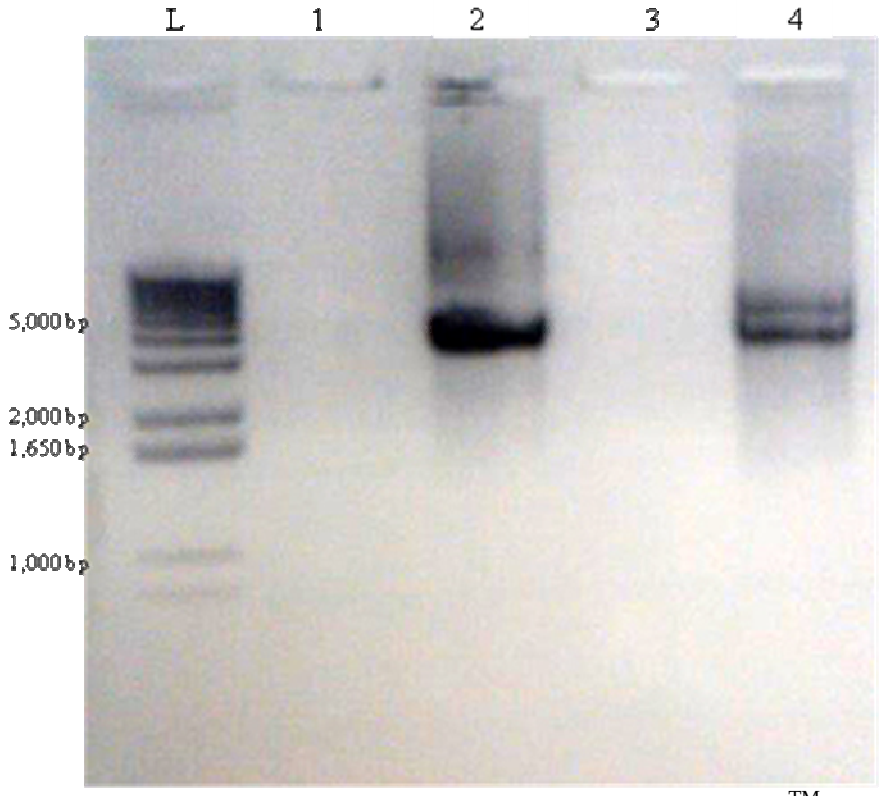

Figure 12. Overlap PCR of *AGO1* and *AGO2* with AccuPrime^{TM} Pfx. The 'L' lane contains a DNA ladder labeled with the appropriate base pair sizes. Lane 1 shows the negative control PCR of the *AGO1*+NAT OL-PCR shown in lane 2. Lane 3 shows the negative control for the *AGO2*+HYG OL-PCR shown in lane 4.

Figure 13. Graphic representation of the *Δago1* gene deletion construct. The construct consists of the 5' flank of the *AGO1* gene fused with the nourseothricin resistance marker and the 3' flank of the *AGO1* gene. This schematic represents the synthesized DNA molecule found in Lane 3 of Figure 12.

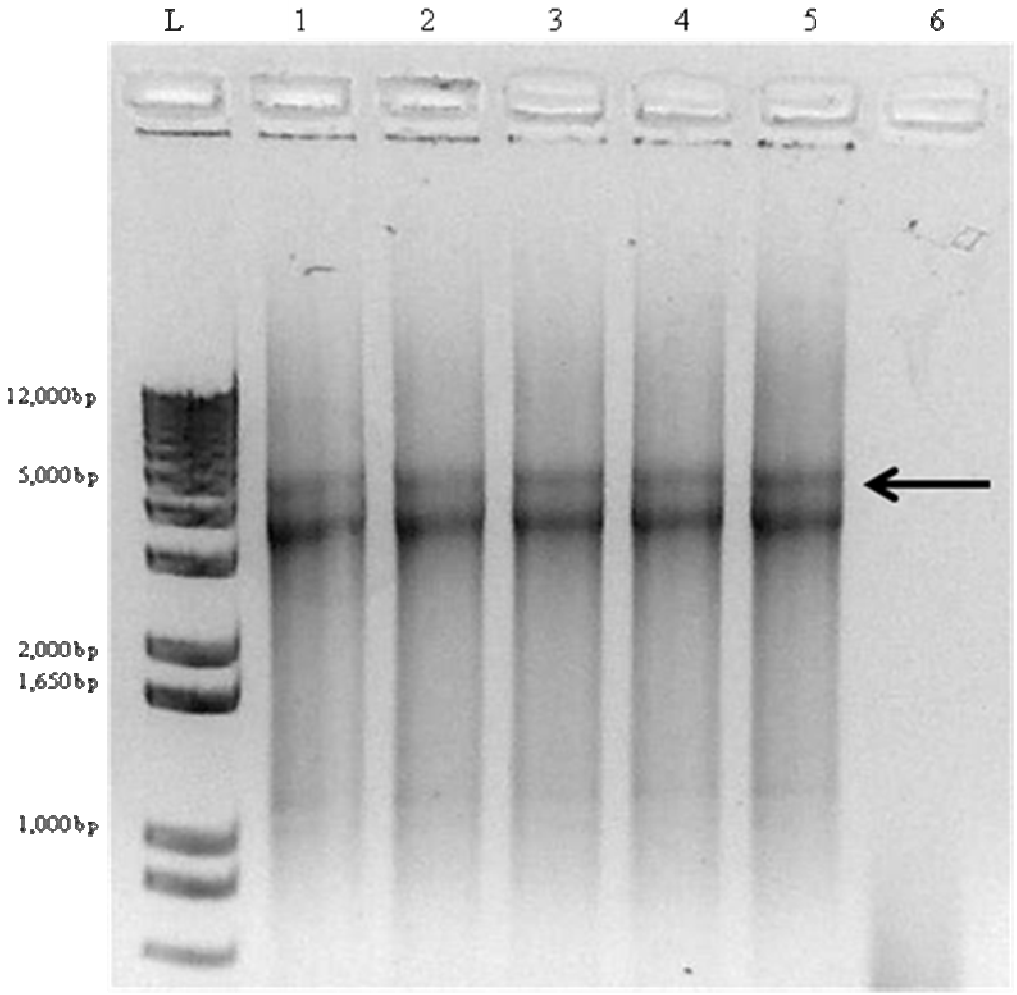

Figure 14. Separate *AGO2*+HYG Overlap PCRs. The 'L' lane contains a DNA ladder marked with the appropriate base pairs sizes. Lanes 1-5 contain *AGO2* overlap PCR products, which are emphasized by the black arrow, at approximately 5kb in size. These amplicons were pooled together and gel purified. Lane 6 shows the negative control for these PCRs.

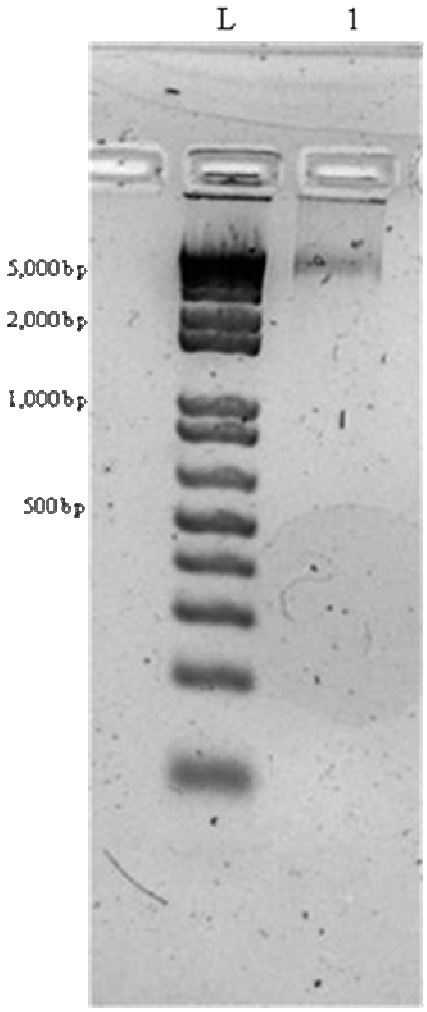

Figure 15. Purified pooled *AGO2*+HYG OL-PCR. The 'L' lane shows a DNA ladder marked with the appropriate sizes in base pairs. Lane 1 contains the *AGO2*+HYG overlap PCR deletion construct and approximately 5kb in size.

Figure 16. Graphic representation of the *Δago2* gene deletion construct. The construct consists of the 5' flank of the *AGO2* gene fused with the hygromycin resistance marker and the 3' flank of the *AGO2* gene. This schematic represents the synthesized DNA molecule found in the right-most lane of Figure 14.

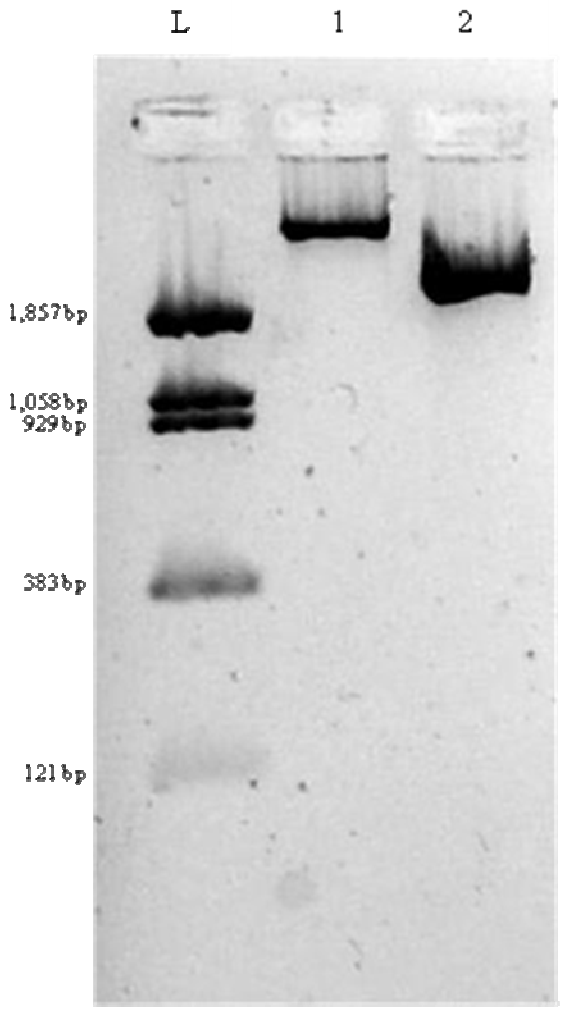

Figure 17. Linearized TOPO-*AGO1* and the G418 resistance marker. The 'L' lane shows a DNA ladder marked with the appropriate band sizes. TOPO-*AGO1* was linearized with MfeI, and is shown in Lane 1. The G418 marker was digested from the TDB546 plasmid using HindIII, and is shown in Lane 2.

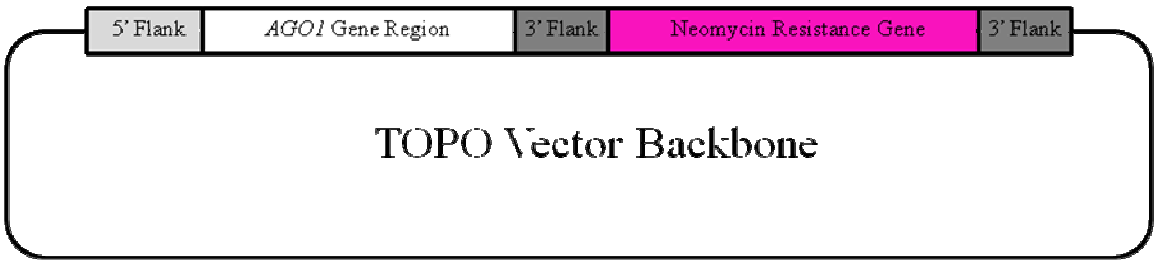

Figure 18. TOPO-*AGO1* Complementation Construct. The above schematic represents the TOPO vector from Invitrogen (Carlsbad, CA.) containing the *AGO1* complementation. A neomycin resistance marker was inserted into the 3'flank of the gene region.

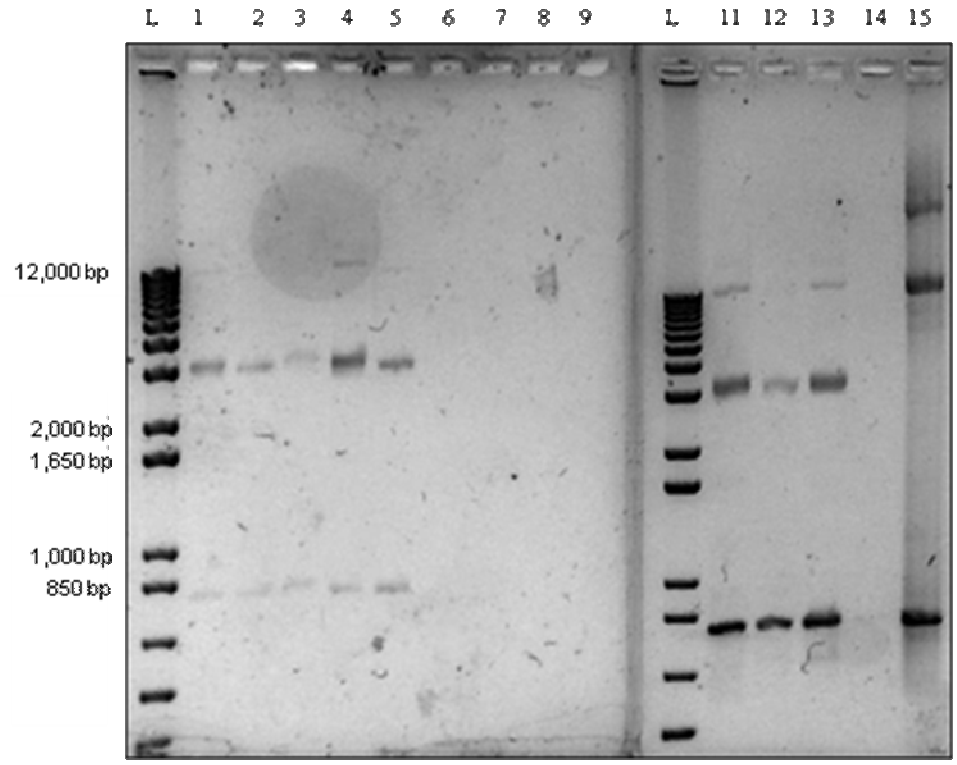

Figure 19. *AGO1* Complementation Construct Colony PCRs. The left-most lane contains a DNA ladder marked with the appropriate base pair sizes. Lanes 1-5 contain neomycin marker bands at approximately 800 base pairs, and another unexplained band at approximately 2,000 base pairs for complementation samples 1-5. Lanes 6-9 were intentionally left unloaded. Lane L contains a duplicate of the DNA ladder. Lanes 11-13 contain an 800 base pair band as well as the unexplained 2000 base pair band for samples 6-8. Lane 14 contains the negative control for this PCR, and lane 15 contains a positive control PCR of the TDB546 plasmid.

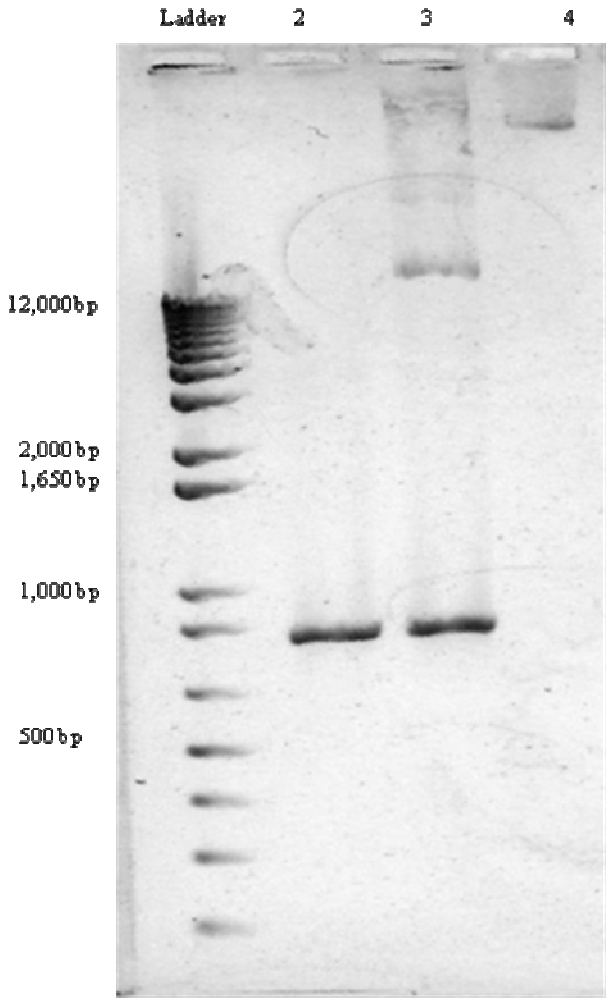

Figure 20. IBB103 Plasmid vs. TDB546 plasmid. The left-most lane contains a DNA Ladder marked with the appropriate base pair sizes. The second lane contains the IBB103 neomycin amplicon. The third lane contains the TDB546 neomycin amplicon. The fourth lane contains the negative control PCR.

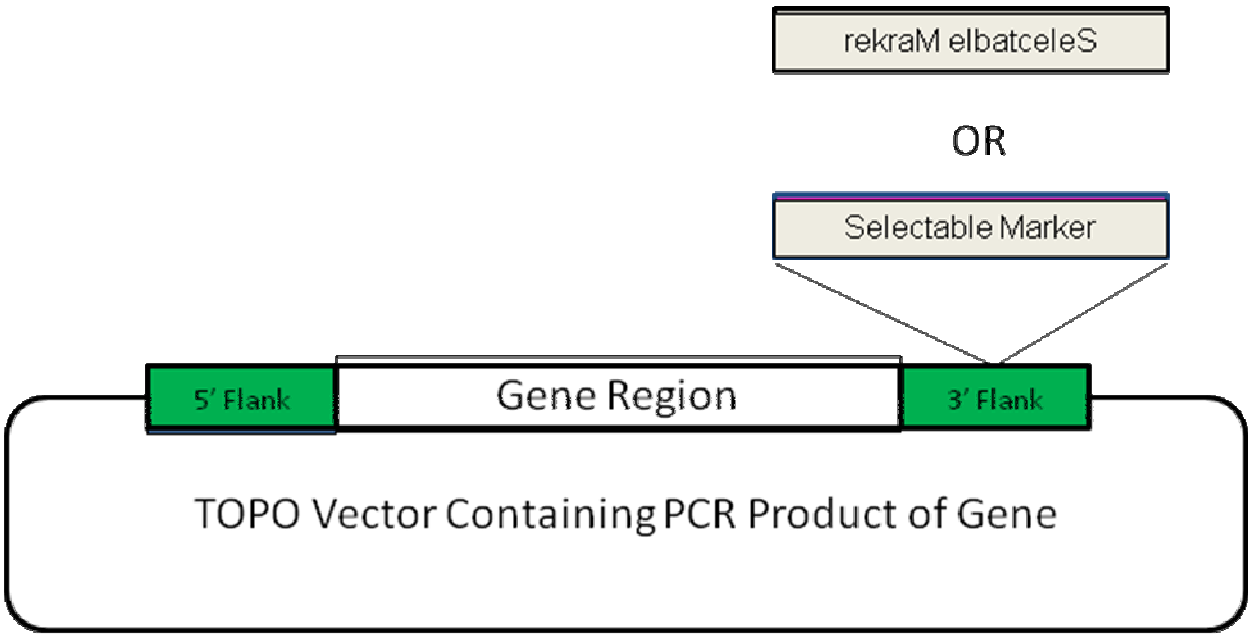

Figure 21. Graphic representation of forward and reverse orientations. The insert can orient into the 3' flank of the gene in one of two ways.

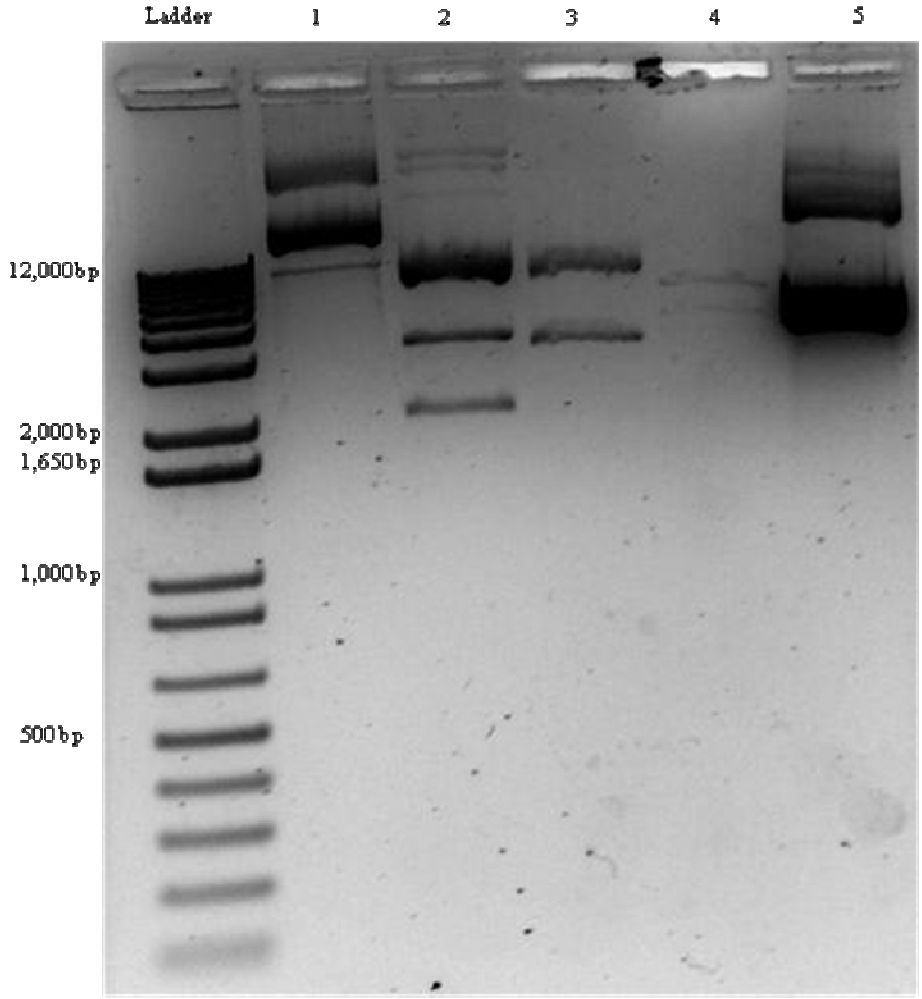

Figure 22. Orientation of *AGO1*(Neomycin) #1 and #2 complementation constructs. The Ladder lane contains the Invitrogen 1Kb Plus Ladder. Lane 1 contains the uncut TOPO-*AGO1*. Lane 2 contains the *AGO1*(Neomycin) #1 plasmid cut with EcoR1 showing three bands, meaning the insert is in the forward orientation. Lane 3 contains the *AGO1*(Neomycin) #2 plasmid also cut with EcoRI showing only two bands, meaning the insert is in the reverse orientation. The fourth lane contains the TOPO-*AGO1* plasmid cut with EcoRI, and the fifth lane shows the uncut TDB546 plasmid.

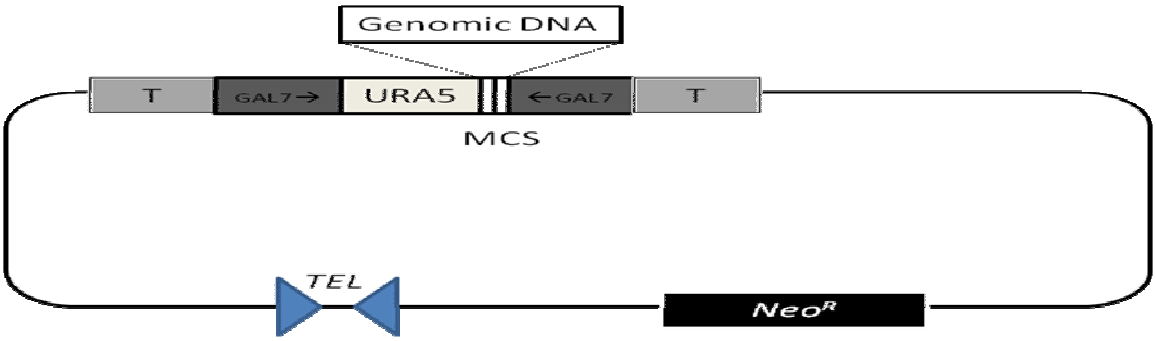

Figure 23. The RNAi Plasmid. This plasmid contains two *GAL7* promoters that transcribe the *URA5* gene and a random piece of genomic DNA from both directions which produce dsRNA. The *GAL7* promoters are flanked by terminators (T). This plasmid also contains a neomycin resistance marker for selection, and telomeres that stabilize the extrachromosomal DNA in *Cryptococcus neoformans*

Table 1. *AGO1*, *AGO2*, *DICER1*, *DICER2* gene lengths and loci. The table below describes the names, abbreviations, loci, and sizes of the four genes which were target for deletion in this study.

Gene Name	Gene Abbreviation	Gene Locus	Gene Size (bp)
Argonaute 1	*AGO1*	CNJ00490	6080
Argonaute 2	*AGO2*	CNJ00610	6130
Dicer 1	NA	CNC03670	5399
Dicer 2	NA	CNC03680	5080

Table 2. *AGO1* knockout primers used for overlap PCR formation of deletion constructs of the Argonaute 1 gene.

Description	Name	Primer Sequence
5' Out	IBO264	5'-CAGGTAATGGCTCGAGCCTTCAAAG-3'
5' In	IBO265	5'-CTTGGGAGCCGTAGGTTTGATAC-3'
3' Out	IBO266	5'-GAGGCTACTACCGTTAAGAGGTTG-3'
3' In	IBO267	5'-CGCGCTATCTCGGAGGAGAAC-3'
5' Selection	IBO503	5'-CAGCCGCAACCCACAGTGAAATCCAGGAAACAGCTATGACCATG-3' Ŧ
Selection 5'	IBO504	5'-CATGGTCATAGCTGTTTCCGGATTTCACTGTGGGTTGCGGCTG-3' Ŧ
3' Selection	IBO271	5'-GAGGAGTCTACAGCCGAGTTCGTTGTAAAACGACGGCCAGTG-3' Ŧ
Selection 3'	IBO270	5'-CACTGGCCGTCGTTTTACAACGAACTCGGCTGTAGACTCCTC-3' Ŧ

Ŧ denotes primers which were PAGE Purified

Table 3. *AGO2* knockout primers used for overlap PCR formation of deletion constructs of the Argonaute 2 gene.

Description	Name	Primer Sequence
5' Out	IBO205	5'-GATCGCCATGAGAAACCTGTC-3'
5' In	IBO206	5'-CGCAGGTAATACACTATCTG-3'
3' Out	IBO207	5'-CTTGGAGGTGGCAGGGCCAAG-3'
3' In	IBO208	5'-CTGCCTCCAAGTTCGCCGTC-3'
5' Selection	IBO209	5'-GAAGGAATCCTCTGTCGGCAAGCAGGAAACAGCTATGACCATG-3' Ŧ
Selection 5'	IBO210	5'-CATGGTCATAGCTGTTTCCTGCTTGCCGACAGAGGATTCCTTC-3' Ŧ
3' Selection	IBO502	5'-CTGGAACAGCGACGTATGTGACGGTTGTAAAACGACGGCCAGTG-3' Ŧ
Selection 3'	IBO501	5'-CACTGGCCGTCGTTTTACAACCATCACATACGTCGCTGTTCACG-3' Ŧ

Ŧ denotes primers which were PAGE Purified

Table 4. Primer Concentrations used in the first round of all OL-PCRs

Gene	Primer Name	Primer Alias	Stock Concentration	Reaction Concentration
AGO1	IBO264	5' Out	32.5 µM	0.65 µM
AGO1	IBO266	3' Out	35.8 µM	0.716 µM
AGO1	IBO270	Selection 3'	50 µM	1 µM
AGO1	IBO271	3' Selection	50 µM	1 µM
AGO1	IBO503	5' Selection	50 µM	1 µM
AGO1	IBO504	Selection 5'	50 µM	1 µM
AGO2	IBO205	5' Out	30.5 µM	0.61 µM
AGO2	IBO207	3' Out	22 µM	0.44 µM
AGO2	IBO501	Selection 3'	50 µM	1 µM
AGO2	IBO502	3' Selection	50 µM	1 µM
AGO2	IBO209	5' Selection	50 µM	1 µM
AGO2	IBO210	Selection 5'	50 µM	1 µM

Table 5. Primer Concentrations used in the second (overlap) round of all OL-PCRs

Gene	Primer Name	Primer Alias	Stock Concentration	Reaction Concentration
AGO1	IBO265	5' In	40.1 µM	0.80 µM
AGO1	IBO267	3' In	32.9 µM	0.65 µM
AGO2	IBO206	5' In	24.5 µM	0.49 µM
AGO2	IBO208	3' In	33.3 µM	0.66 µM

Table 6. Primer sequences used in colony PCR.

Description	Name	Sequence
AGO1 Forward Primer	IBO272	5'-CTCCGATTCACCAAAGCCGGC -3'
AGO1 Reverse Primer	IBO273	5'-CGTCTCGGAAGAACAAGATGC-3'
AGO2 Forward Primer	IBO213	5’-GGACGCTGCTAAGCTTCTC-3’
AGO2 Reverse Primer	IBO214	5’-GTAGGGGGTTTAGCAGCTTCC-3’

Table 7. Representation of a typical ligation reaction. Each ligation consisted of a control ligation containing only the vector, and two test groups with increasing amounts of insert incubated with the ligation. Reactions were incubated at room temperature for 16 hours.

	Vector Only	Reaction A	Reaction B
Vector	2 µL	2 µL	2 µL
Insert	-	3 µL	6 µL
10X T4 DNA Ligase Buffer	2 µL	2 µL	2 µL
T4 DNA Ligase- 2000 Units	1 µL	1 µL	1 µL
Sterile Water	15 µL	12 µL	9 µL
Total	20µL	20µL	20µL

Table 8. Primers used for confirming G418 Complementation Constructs. The table below gives the sequences for the G418 Sense and Antisense primers used in this study to detect complementation in a colony PCR technique.

Description	Name	Sequence
G418 Forward Primer	g418-s	5'-GATCCATGATTGAACAATAT-3'
G418 Reverse Primer	g418-as	5'-TTGGACCAGATGTACCCCGCG-3'

Table 9. Complete database of frozen *E. coli* containing plasmids. The table describes the strain name, the plasmid contained in the strain, the selectable markers that can be used, the original bacterial strain type, and the date frozen.

Strain Name	Plasmid	Selectable Marker	Bacterial Strain
ACB001	pCR2.1-AGO1 (#1)	AMP, KAN	NEB DH5α
ACB002	pCR2.1-AGO1 (#2)	AMP, KAN	NEB DH5α
ACB003	pCR2.1-AGO1 (#3)	AMP, KAN	NEB DH5α
ACB004	pCR2.1-AGO1 (#4)	AMP, KAN	NEB DH5α
ACB005	pCR2.1-AGO1 (#5)	AMP, KAN	NEB DH5α
ACB006	pCR2.1-AGO1 (#6)	AMP, KAN	NEB DH5α
ACB007	pCR2.1-AGO1 (#7)	AMP, KAN	NEB DH5α
ACB008	pCR2.1-AGO1 (#8)	AMP, KAN	NEB DH5α
ACB009	pCR2.1-AGO1 (#9)	AMP, KAN	NEB DH5α
ACB010	pCR2.1-AGO1 (#10)	AMP, KAN	NEB DH5α
ACB011	pCR2.1-AGO1 (#11)	AMP, KAN	NEB DH5α
ACB012	pCR2.1-AGO1 (#12)	AMP, KAN	NEB DH5α
ACB013	pCR2.1-AGO1 (#13)	AMP, KAN	NEB DH5α
ACB014	pCR2.1-AGO1 (#14)	AMP, KAN	NEB DH5α
ACB015	pCR2.1-AGO1 (#15)	AMP, KAN	NEB DH5α
ACB016	pCR2.1-AGO1 (#16)	AMP, KAN	NEB DH5α
ACB017	pCR2.1-DICER2 (#17)	AMP, KAN	NEB DH5α
ACB018	pDrive-Dicer2 (#26)	AMP, KAN	Invitrogen OneShot
ACB019	pDrive-Dicer2 (MP14)	AMP, KAN	Invitrogen OneShot
ACB020	pDrive-Dicer2 (MP14)	AMP, KAN	Invitrogen OneShot
ACB021	pDrive-Dicer2 (MP14)	AMP, KAN	Invitrogen OneShot
ACB022	pDrive-Dicer2 (MP14)	AMP, KAN	Invitrogen OneShot
ACB023	pDrive-Dicer2 (MP14)	AMP, KAN	Invitrogen OneShot
ACB024	pDrive-Dicer2 (MP14)	AMP, KAN	Invitrogen OneShot
ACB025	pCR2.1-AGO1 (#13)	AMP, KAN	NEB DH5α
ACB026	pCR2.1-AGO1 (IB#2)	AMP, KAN	NEB DH5α
ACB027	pCR2.1-AGO1 (IB#3)	AMP, KAN	NEB DH5α
ACB028	pCR2.1-AGO2 (#14)	AMP, KAN	NEB DH5α
ACB029	pCR2.1-AGO2 (#16)	AMP, KAN	NEB DH5α
ACB030	pCR2.1-AGO1(Neo)#1	AMP, KAN	NEB DH5α
ACB031	pCR2.1-AGO1(Neo)#2	AMP, KAN	NEB DH5α

DISCUSSION

Cryptococcus neoformans is the causative agent of cryptococcosis in immunocompromised patients (Buchannan & Murphy, 1998). Treatments for this disease are generally not effective and no advancements in antifungal therapies have been made in over a decade (Odds, Brown, & Gow, 2003). The purpose of this study was to create deletion strains of *C. neoformans* that lacked the *AGO1, AGO2, DICER1,* and *DICER2* genes- four of the genes that are believed to play a role in the RNAi biochemical pathway. Because PCRs of only the *AGO1, AGO2,* and *DICER2* genes were successful (Figure 8), the study was continued using only the *AGO1* and *AGO2* genes. Since *DICER1* did not successfully amplify by PCR, deleting *DICER2* by itself would not necessarily change the RNAi phenotype. Redundancy in the functions of the two *DICER* genes may lead to residual RNAi activity remaining in the *Δdicer2* strains. Deletion constructs for both the *AGO1*and *AGO2* genes were successfully created using the overlap PCR method described previously (*AGO1* Figure 13; *AGO2* Figure 16). However, these PCRs required much optimization for many reasons.

Many things can cause a PCR to fail. Some conditions that may lead to the production of unwanted amplicons include high concentrations of template DNA, high concentrations of oligonucleotides, and the annealing temperature used in a given reaction. It has been found that template concentration can have a major impact on PCR performance (Renshaw-Gegg and Guiltinan, 1996). An excess amount of genomic DNA

of high molecular weight can often lead to inefficient amplification of PCR fragments. Annealing temperatures, if not optimized, can generate non-specific amplification due to non-specific binding of the primers, i.e., the primers are capable of partially binding to multiple regions within the template, thus amplifying unwanted portions of that template. Changing the template and primer concentration, as well as performing gradient PCR allowed for both the amplification of the preliminary PCRs (Figure 6a). The same optimization allowed the amplification of three of the four gene regions in their entirety (Figure 8). Because of this, the conclusion was made that amplicon size was not an issue for the DNA polymerases being used. DNA polymerases may also have an impact upon PCR efficiency. In this study, several different Taq polymerases were used. The best result was obtained with the Pfx Accuprime Taq Polymerase, a proofreading polymerase marketed by Invitrogen (Carlsbad, CA).

The overlapping regions generated by the primers are of paramount importance to the overlap PCR procedure. Although Davidson and his colleagues (Davidson et al., 2002) had used desalted but unpurified primers for generating overlap PCR fragments, it appears that purified primers may yield better results in certain cases. In order to increase the integrity of the overlapping regions of the three amplicons (which is generated by the primers), primers were PAGE-purified. PAGE-purified primers allow for any incompletely synthesized primers to be removed from the stock, thus leaving only the complete, full primer. Interestingly, no OL-PCR using non-purified primers gave any overlap result, although PAGE-purification is not generally necessary for OL-PCR (Davidson et al., 2002). The caveat to this is that I did not use the non-purified primers with the Pfx Accuprime Taq polymerase in a PCR.

The initial attempt at an overlap PCR with the purified primers yielded very little DNA (Figure 10). To correct this, an alternative Taq polymerase was used that generated a much higher yield of the three preliminary PCRs, and ultimately, a greater quantity overlap band for the *AGO1* deletion construct. The *AGO2* deletion construct, however, was always seen at a lower yield than the *AGO1*, and betaine seemed to inhibit the PCR. Rather than wasting many expensive reagents in an attempt to optimize the quantity of the *AGO2* deletion construct, another approach was taken. Five individual PCRs were run (Figure 14) and the overlap products (the *AGO2* deletion constructs) from each PCR were pooled together and purified in order to generate enough DNA for an attempt at biolistics (Figure 15). The *AGO2* deletion construct was not used as a template for PCR to increase DNA for two main reasons. The first reason concerned the integrity of the primer binding sites for the PCR, and the other concerned the overall integrity of the sequence of the amplicon. Any mistake made by the polymerase could have led to potential inactivity of the selectable marker. This is not a concern for the original *AGO2* deletion construct, but copying a copy could lead to the buildup of erroneous bases in the DNA amplicon.

Because deletion constructs were successfully created for both the *AGO1* and *AGO2* genes, complementation constructs were attempted as well. Complementation is a necessary step in any gene deletion study. Deletion constructs allow for the creation of deletion strains leading to the study of the function of any given gene. However, complementation constructs can be used to rescue a deletion strain by reintroducing the wild type gene, therefore confirming that the results gleaned from studies of the deletion strain are directly related to the deletion of the target gene and not related to some other

phenomenon or some step in the deletion process (an artifact of the transformation and deletion protocol).

Construction of the complementation vector for the *AGO1* deletion was successfully completed using traditional molecular cloning methods that allowed the insertion of a neomycin marker into the TOPO-*AGO1* cassette. Because there was an unexpected band in these PCRs (Figure 20), another PCR was run to address the issue (Figure 21). The IBB103 plasmid that also contains the neomycin resistance marker gave the same banding pattern as the TDB546 plasmid using the primers located in Table 8. Because these two separate plasmids gave the same result using exact PCR conditions, it was concluded that the eight colonies screened as potential *AGO1* complementation constructs do contain the neomycin resistance marker in the correct location. Figure 22 shows the orientation of the *AGO1* complementation plasmids of the *AGO1*(Neomycin) #1 and #2 plasmids, confirming that *AGO1*(Neomycin) #1 plasmid contains the insert in the forward orientation and that *AGO1*(Neomycin) #2 contains the insert in the reverse orientation. For our purposes, restriction enzyme digests proved more simple and more high-throughput than sequencing, as these plasmids are over 10 kb, a size that is not ideal for DNA sequencing reactions. These nascent plasmids are available for complementation of the deletion strains of *C. neoformans* upon their creation.

Complementation of *AGO2* was not possible during the scope of this study because the TOPO-*AGO2* plasmid does not contain a single restriction enzyme site near the stop codon, making it impossible to add a complementation marker into the plasmid. Another methodology to perform this task is proposed in the Future Work section.

Deletion constructs of both the *AGO1* and *AGO2* genes were submitted for biolistics at the Doering Lab at Washington University in St. Louis, MO. However there is no estimated time for the biolistics to be completed. This issue will be further discussed in the Future Work section.

FUTURE WORK

Transformation of *Cryptococcus* via Biolistics to Produce Deletion Strains

As the deletion constructs are already prepared, the next step in this project would be to transform *C. neoformans* cells with these constructs to make the corresponding deletion strains. *Crptococcus neoformans* can be transformed by two processes: electroporation or biolistics. However, biolistics is the preferred choice if integration of the DNA constructs is required. Incorporation of transformed sequences into the genome occurs at a very low frequency by the process of electroporation. Recent work has shown that the addition of telomeric sequences to the plasmid increases transformation efficiency by electroporation by orders of magnitude (Indrani Bose, personal communication 2009) but that these plasmids mostly remain extrachromosomal. The overlap PCRs can be used directly in the biolistics process because, for reasons that remain unclear, *C. neoformans*, preferentially accepts linear DNA over circular plasmid DNA (Toffaletti and Perfect, 1993, Davidson et al., 2000).

The deletion constructs (generated by OL-PCR) will be transformed into the KN433 *MAT* **α** and KN433 *MAT* ***a***-Serotype D strains of *Cryptococcus neoformans* (Nielsen & Heitman, 2005). KN433 *MAT* **α** will be transformed with the *ago1::NAT* construct, while the *ago2::HYG* constructs will be transformed into the KN433 *MAT* ***a*** strain. Transformed cells will be grown on plates with the appropriate drug (nourseothricin for *ago1* and hygromycin for *ago2*) in an incubator at 30°C for 3-5 days

for growth of individual fungal colonies (adapted from Toffaletti and Perfect, 1993). Due to equipment and spatial limitations, all biolistics will need to be performed at the Doering Lab, 10-230 McDonnell Pediatrics Building, Washington University Medical Campus, St. Louis, MO., 63130.

Creating *AGO1* and *AGO2* Double Deletions

KN433 *MAT* **α** and KN433 *MAT* ***a*** are congenic strains of *Cryptococcus neoformans*, differing only from one another at the *MAT* locus, allowing them to mate. Therefore other than the *MAT* locus, they should differ only in the particular argonaute gene deletion.

Double deletions can be obtained by mating the *ago1::NAT* KN433 *MAT* **α** and the *ago2::HYG* KN433 *MAT* **a** strains on V8 agar as previously described (Nielsen & Heitman, 2005). The F_1 progeny can then be grown on selectable YPD+NAT+HYG agar by incubating in an incubator at 30°C for 3-5 days for fungal growth. The surviving colonies would be tested for the presence of the correct deletion (as described below).

Screening Transformants for Gene Deletion

All the surviving colonies from the above transformations that are nourseothricin and hygromycin resistant could be selected for genomic preparation. Colonies would be grown overnight in 5 mL of YPD broth. Genomic preparations of fresh cultures would be performed as previously described (in the Methods and Materials Section). The genomic DNA then can be used as a template in PCR reactions to amplify the NAT marker (for the *Δago1* strains) and the HYG marker (for the *Δago2* strains), and these PCRs need to be analyzed by DNA gel electrophoresis. The colonies with the correct PCR results will have to be checked by Southern hybridization for insertion of the

construct in a single locus of the genome replacing the wild type gene of choice alone. This is because *Cryptococcus* cells often incorporate exogenous DNA at several places in their genome.

Creation of an *AGO2* Complementation Construct

Because the TOPO-*AGO2* plasmid lacks a single restriction enzyme site within 300 base pairs from the stop codon (which is the distance arbitrarily taken to be necessary for termination of transcription), molecular cloning techniques used for the *AGO1* complementation will not suffice. To generate an *AGO2* complementation, a PCR-based strategy will need to be employed to amplify the entire *AGO2* gene with 1 kb 5' untranslated region (UTR) and 3' UTR. A selectable marker (such as the neomycin resistance gene) can also be amplified and biolistically co-transformed with the *AGO2* gene fragment. This kind of co-transformation strategy has been previously used to obtain transformants in *C. neoformans* (Goins, Gerik, and Lodge, 2006). After transformation, cells will be screened for the presence of the neomycin resistance gene (by plating on YPD+G418 plates) and the absence of the hygromycin resistance gene. In addition, the colonies with the right combinations of markers should also be tested by PCR to ensure the *AGO2* ORF is in the correct context (i.e., to ensure that the *AGO2* gene was inserted in place of the HYG marker, in the correct chromosome). Because the G418 PCR was co-transformed, it is not necessarily associated with the gene, and could have been inserted someplace else, possibly disrupting other genes. To correct this, the *AGO2* (G418) *MAT*-***α*** strain will need to be mated with a wild type *MAT*-***a*** strain. The F_1 spores can then be tested for the presence of G418 via PCR. After the G418 has been removed

from the genome, the *Δago2* will have successfully been complemented with the wild type gene.

Testing for the Presence of RNAi

The transformed *Cryptococcus* will then be tested for the ability to perform RNAi. To do this, the RNAi plasmid IBB103 (Figure 23) will be transformed into the *Δago1*, *Δago2*, and Δ*ago1*Δ*ago2* mutant strains of *C. neoformans*. This plasmid contains a neomycin resistance marker and an RNAi cassette that allows for the production of dsRNA corresponding to the sequence of the *URA5* gene fragment cloned in between two *GAL7* promoters in opposite directions (Figure 24). If RNAi is functioning properly, the presence of the dsRNA from the *URA5* gene fragment in the plasmid would lead to silencing of the *URA5* gene and no uracil production. This would make the strain a *ura5* mutant without any change occurring to the genomic DNA. However, if RNAi has been disrupted, the *URA5* gene would not be silenced, completing uracil biosynthesis.

Deletion strains will be plated on minimal media that contains neomycin, uracil, galactose and 5-fluoro-orotic acid (5FOA). If cells did not receive the plasmid, they will be unable to grow due to the presence of neomycin in the media. Only cells that received the plasmid will be able to grow. The galactose in the media will activate the *GAL7* promoters and the *URA5* dsRNA will be expressed. Without RNAi, the protein encoded by *URA5* will use the 5FOA as a substrate, resulting in 5-fluoro-uracil, a toxic pyrimidine killing the cells. Alternatively, if RNAi is functioning, cells will grow because RNAi will silence the *URA5* gene and the cells will use the uracil provided in the media.

To test the complementation constructs, a variant of the RNAi plasmid will be used that contains a hygromycin resistance marker in lieu of the neomycin marker, as

neomycin was used to create the complementations. Deletion strains will be plated on minimal media that contains hygromycin, uracil, galactose and 5FOA. The test for these complementation constructs should be carried out in the same manner as the neomycin deletion tests (discussed above).

Other Studies Involving *Δago1* and *Δago2* Mutants

Further studies would be performed to classify the status of known factors of virulence in *C. neoformans* such as the formation of the polysaccharide capsule, thermotolerance and mannitol synthesis. Also, these mutants, if incapable of RNAi, would be used in an end-tail murine virulence study to determine if the absence of RNAi abrogates virulence in these fungi.

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APPENDICES

Appendix I

PCR Reagents

Sterile Water- Sterile water was prepared by obtaining mmH_2O using an ELGA Classic Ultrapure Water Purification System (High Wycombe, UK) and autoclaving the water at 15 psi and 121°C for 30 minutes under slow exhaust of a STERIS Remaunfactured 3021 Gravity Steam Sterilizer (Life Sciences Group, Mentor, OH).

5X CES Stock- A 1 mL, 5X stock of the combinatorial enhancement solution for overlap PCR was prepared by combining 540 µL of a 5M betaine stock, 6.7 µL of 1M dithiothreitol (DTT), 67 µL of DMSO, and 55 µL of a 1µg/mL BSA stock with 331.3 µL of sterile mmH_2O. The stock solution was aliquoted into four aliquots and stored at -20°C.

Appendix II

Antibiotic Stock Solution Preparation

1000X Ampicillin Stock- ampicillin stocks were created by adding 500 mg of ampicillin sodium salt (ACROS Organics, Morris Plains, NJ) to 10 ml of mmH_2O. The solution was filter sterilized using a 10 ml sterile syringe and a FisherBrand 0.22 µm sterile filter (Fisher Scientific, Pittsburg, PA) This 1000X ampicillin stock was divided into ten 1 ml aliquots and stored frozen at -20°C.

1000X Kanamycin Stock- kanamycin stocks were created by adding 500 mg of kanamycin monosulfate (Fisher Scientific, Pittsburg, PA) to 10 ml of mmH_2O and filter sterilized using a 10 ml sterile syringe and a FisherBrand 0.22 µm sterile filter (Fisher Scientific, Pittsburg, PA) This 1000X kanamycin stock was divided into ten 1 ml aliquots and stored at -20°C.

Appendix III

Preparation of Electrophoresis Buffers and Agarose

Tris-Borate-EDTA (TBE) Buffer- A 10X stock solution of TBE buffer was prepared by dissolving 108g of Tris base and 55g of Boric Acid in 200 µL of a 0.5M EDTA solution and enough diH_2O to bring the final volume to 1 L. To dilute this solution for use as an electrophoresis running buffer, 100 mL of the stock was diluted using 900 mL of mmH_2O.

Loading Dye- A 6X loading dye was prepared using 10mM Tris HCl (pH=7.5) + 1mM 0.5M EDTA in 50% glycerol. To this solution, a few grains of bromophenol blue were added. The loading dye was stored in 1 mL aliquots at -20°C.

Agarose Gel- Agarose solution was prepared in bulk and stored in a bottle on the benchtop until needed as follows: 3 grams of Electrophoresis Grade Agarose (Fisher Scientific, Pittsburg, PA) was added to 300 ml of a 1X TBE Buffer solution. The solution was microwaved until the agarose was completely dissolved. The agarose solution was allowed to cool to 60°C and 15 µL of ethidium bromide was added. When needed, this "stock" solution was melted in the microwave and poured into the appropriate gel casting tray.

Appendix IV

Bacterial Growth Media Preparation

Luria-Bertani Media Plates + Antibiotic- These media plates were produced by dissolving 25 g of LB Broth (ACROS Organics, Morris Plains, NJ) and 15 g of Bacto™Agar (Becton-Dickson Inc., Franklin Lakes, NJ) in 1 L of mmH_2O. The media was autoclaved at 15 psi and 121°C for 30 minutes under slow exhaust of a STERIS Remaunfactured 3021 Gravity Steam Sterilizer (Life Sciences Group, Mentor, OH). Sterile media was slowly stirred and allowed to cool to approximately 37°C, and 1 ml of the appropriate 1000X antibiotic stock was added. The media was stirred for an additional minute, and 15 mL plates were poured from the media. Plates were allowed to solidify and excess condensation dried out before being moved to 4°C for storage.

Luria-Bertani Liquid Broth- Luria-Bertani broth was prepared by dissolving 25 g of LB Broth (ACROS Organics, Morris Plains, NJ) in 1 L of mmH_2O and autoclaving for 30 minutes at 121°C under 15 psi pressure in a STERIS Remaunfactured 3021 Gravity Steam Sterilizer (Life Sciences Group, Mentor, OH). Sterile LB Broth was stored at room temperature.

Appendix V

Yeast Growth Media Preparation

Yeast-Peptone Dextrose (YPD) Media Plates- YPD media plates were prepared by dissolving 50 g of YPD Broth (Sigma-Aldrich, Inc., St. Louis, MO) and 15 g of Bacto™Agar (Becton-Dickson Inc., Franklin Lakes, NJ) in 1 L of mmH_2O. The media was autoclaved at 15 psi and 121°C for 30 minutes under slow exhaust of a STERIS Remaunfactured 3021 Gravity Steam Sterilizer (Life Sciences Group, Mentor, OH). 15 mL Petri plates were poured from this media. Plates were allowed to solidify and dry before they were placed at 4°C for storage.

Yeast-Peptone Dextrose (YPD) Broth- YPD broth was prepared by dissolving 50 g of YPD Broth (Sigma-Aldrich, Inc., St. Louis, MO) in 1 L of mmH_2O. Prior to sterilization, the media was divided into 10 100 mL aliquots. These aliquots were autoclaved at 15 psi and 121°C for 30 minutes under slow exhaust of a STERIS Remaunfactured 3021 Gravity Steam Sterilizer (Life Sciences Group, Mentor, OH). Sterile LB media was stored at room temperature.