[*This is a protocol for publication in CPMC. There are 7 figures and 2 tables included. A required PMC file is also included.]

Gene Editing in Dimorphic Fungi Using CRISPR/Cas9

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Abstract

Dimorphic fungi in the genera *Blastomyces*, *Histoplasma*, *Coccidiodes* and *Paracoccidiodes* are important human pathogens that affect human health in many countries throughout the world. Understanding the biology of these fungi is important for the development of effective treatments and vaccines. Gene editing is a critically important tool for research into these organisms. In recent years, gene targeting approaches employing RNA-guided DNA nucleases, such as clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated (Cas) nuclease 9, have exploded in popularity. Here we provide a detailed description of the steps involved in applying CRISPR/Cas9 technology to the dimorphic fungi, with *Blastomyces dermatitidis* in particular as our model fungal pathogen. We discuss the design and construction of single guide RNA (sgRNA) and Cas9-expressing targeting vectors (including multiplexed vectors), as well as introduction of these plasmids into *Blastomyces* using *Agrobacterium*-mediated transformation (AMT). Finally, we cover the outcomes that may be expected in terms of gene-editing efficiency and types of gene alterations produced.

Keywords

Gene targeting, CRISPR/Cas9, *Agrobacterium*-mediated transformation, *Blastomyces*, dimorphic fungi

Introduction

Thermally dimorphic fungi represent a group of human fungal pathogens that can cause serious systemic infections in both healthy and immunocompromised individuals. Example species include *Blastomyces dermatitidis*, *Coccidioides immitis*, *Histoplasma capsulatum*, *Paracoccidiodes brasiliensis* and several others. These fungi grow as saprophytic molds at ambient temperatures (e.g. 25°C), producing spores that can be inhaled and convert to yeast at the elevated body temperature of mammalian hosts (e.g. 37°C). If localized immune responses in the lung fail to contain the infection, dissemination to multiple organ systems can result in serious organ dysfunction and in some cases, even death. Currently, no vaccines against any dimorphic fungus are available. While antifungal drugs such as azoles and amphotericin can be effective, antifungal resistance is a growing concern. Thus, identifying virulence factors and understanding immune responses to these fungi are important steps toward vaccine and antifungal drug development efforts. Gene disruption is an important means to test the role of specific fungal components in infection.

Prior efforts at targeted genetic modification of the dimorphic fungi have employed RNA interference and recombination-based gene targeting (Krajaejun et al., 2007; Sullivan et al., 2002; Brandhorst et al., 1999; Woods et al., 1998; Youseff and Rappleye, 2012; Xue et al., 2009). RNA interference can reduce but often does not completely suppress target gene expression. Homologous recombination occurs at a low frequency in *Blastomyces*, so gene disruption that relies on this method exhibits low efficiency, necessitating the screening of large numbers of transformants in order to find the desired mutants. By contrast, CRISPR/Cas9 approaches have proven to be effective and versatile methods of gene editing in a wide range of organisms (Doudna and Charpentier, 2014). CRISPR/Cas9 gene editing requires a single guide RNA (sgRNA) with complementarity to the target gene sequence that directs the Cas9 nuclease to generate a double-stranded DNA break at the locus of interest. During the cellular process of repairing the break, small base insertions or deletions occur, generating frame-shifted nonsense proteins and loss of gene function. We have recently succeeded in applying this technology to the dimorphic fungus, *Blastomyces dermatitidis* (Kujoth et al., 2018).

In many model organisms, the sgRNA and Cas nuclease components may be provided as ribonucleoprotein complexes or expressed from appropriate plasmid vectors, depending upon the ease of introducing these materials into the cells of interest. In the case of the dimorphic fungi, transfection methods such as electroporation or lipofection may not provide optimal means for introducing foreign materials into the cell, which limits the ability to utilize preformed sgRNA/Cas nuclease complexes. Instead, transformation of these fungi with plasmid vectors expressing Cas nuclease and sgRNA transcripts is a more practical approach.

Our application of CRISPR/Cas technology to *Blastomyces* builds on the approach developed by Nødvig *et al.* (2015) for gene editing in filamentous fungi, such as *Aspergillus*. It relies on a binary targeting vector containing: (i) an expression cassette of *Streptococcus pyogenes* Cas9 nuclease, modified to contain a nuclear localization signal and codon optimized for translation in filamentous fungi ; (ii) a sgRNA expression cassette; (iii) the hygromicin B phosphotransferase (hph) gene as a selectable marker; and (iv) left border and right border transfer DNA repeat sequences needed for *Agrobacterium*-mediated transformation (AMT, see below). The sgRNA is expressed from the *Aspergillus nidulans gpdA* promoter (a RNA polymerase II promoter) as a primary transcript flanked by upstream and downstream ribozyme sequences (see Figure 1). These autocatalytic ribozymes self cleave the primary transcript to liberate the internal sgRNA, making it available to complex with Cas9. For multiplexed targeting, the sgRNA cassette is modified to contain multiple ribozyme-flanked sgRNAs within the primary transcript.

[*Place figure 1 near here.]

In order to deliver the targeting vector into *Blastomyces* cells, we employ AMT (Sullivan et al., 2002). Naturally occurring Agrobacteria are found in association with wounded plant tissue and possess a complex system for horizontal gene transfer of growth-promoting genes into adjoining plant cells, resulting in plant tumors known as crown galls. Components of this gene transfer system are encoded on a so-called "tumor-inducing" or Ti-plasmid and include virulence (or *vir*) genes involved in sensing and transducing the hormone signals produced by injured plant tissues, as well as transcription factors and structural components necessary for the construction of the conjugation-like transfer apparatus and the mobilization of a region of the Ti-plasmid called the transfer DNA (T-DNA). The T-DNA region itself carries plant growth-promoting oncogenes and opine genes (amino acid compounds that the *Agrobacterium* can use as a food source), flanked by 25 bp imperfect repeat sequences at its left and right borders that signal the beginning and end of the region to be excised. Importantly from a bioengineering point of view, the T-DNA and the remainder of the Ti-plasmid can be physically separated and the two will function in *trans* for efficient gene transfer. Replacing the contents of the T-DNA with ectopic genes of interest allows the researcher to exploit *Agrobacterium* for gene delivery and this has proven to be a successful strategy in fungi as well as plants (de Groot et al., 1998; Michielse et al., 2005). For our purposes, then, we need a binary vector that can be manipulated in *E. coli* for cloning, maintained in *Agrobacterium* and which possesses the border repeats needed to define the recombinant T-DNA region to be transferred. As we detail in this chapter, our binary targeting vectors carry the Cas9 and sgRNA expressions cassettes in between the border repeats needed to define the recombinant T-DNA region to be transferred. The remaining required components are carried on a "disarmed" Ti-plasmid (so-called because it is devoid of the native oncogenic T-DNA region) provided by the *Agrobacterium* helper strain [*A. tumefaciens* LBA1100 (Beijersbergen et al., 1992)].

We recently combined established binary T-DNA vectors with the components needed for CRISPR targeting and clonal selection in *Blastomyces*. Several examples of the resulting targeting vectors are shown in Figure 1. The target gene-specific 20 bp protospacer region is changed for each novel gene target. Figure 1C shows a targeting vector containing tandem sgRNA cassettes being driven off of a

single promoter for dual gene targeting. Swapping out protospacers is typically done using polymerase chain reaction (PCR) and Gibson assembly cloning. Adaptation of these vectors to incorporate different selectable markers (e.g., neomycin phosphotransferase for G418 resistance) in place of hygromycin-resistance has also been pursued (not shown). Finally, it is important to note that a binary vector expressing Cas9 but lacking any sgRNA expression (pPTS608-Cas9-hyg, Figure 1A) is used as a control to monitor for any unexpected effects of constitutive Cas9 expression in transformed fungal cells. So far, we have seen no ill effects of long term Cas9 expression in any such "Cas9-only" controls compared to wild-type fungal cells.

This protocol details the specific steps that we use to accomplish efficient gene disruption in *Blastomyces* and we hope that this will serve as a guide to expanding the use of CRISPR/Cas in other dimorphic fungi. This protocol contains methods for the selection of protospacers in a gene of interest, the construction of Cas9- and sgRNA-expressing targeting vectors, their introduction into *Blastomyces* cells via *Agrobacterium*-mediated transformation (including a support protocol for preparation of electrocompetent *Agrobacterium*), screening transformants to identify edited clones and freezing such clones for long term storage.

Biosafety Cautions

CAUTION: *Blastomyces dermatitidis* (as used in this protocol in its yeast form) is a Biosafety Level 2 (BSL-2) pathogen. Follow all appropriate guidelines and regulations for the use and handling of pathogenic microorganisms. See Burnett et al. (2009) for more information.

Basic Protocol 1: Construction of CRISPR/Cas9 Targeting Vectors

Construction of targeting vectors involves several stages: (i) choosing specific 20 nt protospacers from your target gene of interest (see Support Protocol 1); (ii) generating the sgRNA expression cassette containing the desired protospacer(s); (iii) cloning the sgRNA expression cassette into the Cas9-expression binary vector backbone; and (iv) introducing the final targeting vector into *Agrobacterium*. The Cas9-only plasmid, pPTS608-Cas9-hyg, serves as the targeting vector backbone for the addition of a sgRNA expression cassette. A pre-existing combined sgRNA- and Cas9-expressing plasmid (e.g., pPTS608-Cas9-hyg-Pra1 sgRNA) is used as a template to generate overlapping PCR fragments, with novel protospacer sequences in the 5' primer tails providing the replacement protospacer portions of the sgRNA (Figure 2). The overlapping PCR products are then combined by Gibson assembly with digested vector backbone.

[*Place figure 2 near here.]

It is important to note that the 5' end of hammerhead ribozyme RNA sequence contains an inverted repeat that is complementary to the first 6 nucleotides of the gene-specific protospacer (Figure 1B). This means that the protospacer and the inverted repeat sequences *both* have to be replaced in every novel targeting vector being constructed.

Materials List

pPTS608-Cas9-hyg plasmid DNA
pPTS608-Cas9-hyg-Pra1 sgRNA plasmid DNA
Custom oligonucleotide DNA primers (Integrated DNA Technologies or other supplier)
Proofreading thermostable DNA polymerase (e.g. Q5 polymerase, New England Biolabs)
10 mM each mixture of dATP, dCTP, dGTP, dTTP
Sterile deionized water
Thermocycler apparatus
Nanodrop Spectrophotometer
Qiaquick Gel Extraction kit (Qiagen) or similar
Gibson Assembly master mix (New England Biolabs)
Electrocompetent *E. coli* (e.g., Electromax DH10B, Life Technologies)
Electroporation apparatus (e.g., Gene Pulser, Bio-Rad)
Electroporation cuvettes, 0.1 cm gap (Bio-Rad)
SOC medium
15 ml snap-cap culture tubes
Luria-Bertani-kanamycin (50 µg/ml) plates and broth
Plasmid DNA miniprep kit (e.g., Qiagen Spin minipreps)
Restriction endonucleases (*Pac*I; *Acc*I or other, as appropriate)
Big Dye Terminator cycle sequencing kit for Sanger sequencing (ThermoFisher)
[*Copy Editor: Please query the authors to include AddGene ID if plasmids are commercially available.]

Steps and Annotations

This protocol assumes familiarity with common molecular cloning techniques, including restriction endonuclease digestion, PCR, Gibson assembly, phenol/chloroform extraction and ethanol precipitation of DNA, transformation of plasmid DNA into *E. coli*, and Sanger sequencing. If needed, the reader is referred to other laboratory manuals (e.g., Slatko et al., 2011; Kramer and Coen, 2006; Seidman et al., 2001; Bloch and Grossmann, 2001; Green and Sambrook, 2012) for additional details.

1. Prepare the vector backbone by digesting pPTS608-Cas9-hyg with *Pac*I to linearize the vector immediately upstream of the *Tef1* promoter driving Cas9 expression (Figure 1A).

pPTS608-Cas9-hyg plasmid DNA	1–2 µg
10X NEB Cutsmart buffer	2 µl
*Pac*I (10U/µl; New England Biolabs)	1 µl
H2O	bring to 20 µl

Incubate for 2 hr at 37°C and check an aliquot for complete digestion using agarose gel electrophoresis. Once fully digested, heat inactivate the enzyme at 65°C for 20 min.

Generally, gel purification of linearized vector is not necessary, but can be done to reduce the potential for uncut vector carryover into the Gibson assembly step. Such carryover can

increase the number of background colonies and will be evident during restriction analysis of putative clones.

2. Design four PCR primers to produce two overlapping fragments, A and B, that contain the novel protospacer and hammerhead ribozyme inverted repeat sequences specific to your target gene of interest.

   Specific examples of these primers are given in Table 1. Only the protospacer and complementary overlap of the hammerhead inverted repeat need to be changed in the FragA R and FragB F primers when designing primers to new target genes of interest. See notes in Table 1. See Support Protocol 1 for discussion of protospacer selection.

[*Place table 1 near here.]

3. Amplify fragments A and B off of a previous targeting vector (e.g., pPTS608-Cas9-Hyg-Pra1 sgRNA) using FragA F/FragA R and FragB F/FragB R primer pairs from step 2 using a proof-reading polymerase (e.g., Q5 polymerase, New England Biolabs).

pPTS608-Cas9-hyg-Pra1 sgRNA vector	1-2 ng
FragA F or FragB F (20 uM stock, C_f=0.5 uM)	1.25 µl
FragA R or FragB R (20 uM stock, C_f=0.5 uM)	1.25 µl
10 mM dNTP mix (C_f=200 uM)	1 µl
5X Q5 buffer	10 µl
Q5 DNA polymerase (2 U/µl)	0.5 µl
Water	bring volume to 50 µl

Cycling parameters:
1 cycle of 98°C for 30 sec
5 cycles of 98°C for 10 sec, 60°C for 20 sec, 72°C for 75 sec
25 cycles of of 98°C for 10 sec, 67°C for 20 sec, 72°C for 75 sec
1 cycle of 72°C for 7 min

Adjust the annealing temperature as needed, based upon the melting temperature of your primers. Because the novel protospacer sequences within the primers will not hybridize to the template, a dual stage cycling profile is used to allow for a lower temperature during the early cycles. Once newly synthesized product has begun to accumulate enough to serve as a template for later cycles, a higher annealing temperature can be used.

4. Purify fragment A and B PCR products (expected sizes of 556 bp and 437 bp, respectively) away from carry-over vector template DNA using ~1% agarose gel electrophoresis and subsequent band extraction. Determine recovered fragment DNA concentration by nanodrop spectrometry.
5. Set up a Gibson assembly reaction to combine the two PCR fragments and linearized targeting vector backbone. Each PCR fragment is typically added in a 3-fold molar excess

relative to the vector backbone. A prototype reaction is given here but the amount or ratios of vector and insert fragments may need to be adjusted in some cases.

*Pac*I-linearized pPTS608-Cas9-hyg vector backbone	200 ng (.02 pmol)
Insert fragment A, gel-extracted	.06 pmol
Insert fragment B, gel-extracted	.06 pmol
2X Gibson Assembly master mix	10 µl
Water	bring volume to 20 µl

6. Incubate at 50°C for 60 minutes. After incubation is completed, place on ice for several minutes.
7. (Optional) After the Gibson assembly reaction incubation has completed, increase the reaction volume by adding 180 µl TE (10 mM Tris, 1 mm EDTA) or water and extract with 200 µl phenol:chloroform, followed by ethanol precipitation.

   The purpose of this additional processing is to remove Gibson assembly enzymes prior to transformation of E. coli. *We have found that this is often not necessary but it may be helpful in problematic cloning circumstances. Carrier molecules such as glycogen (e.g., 5 µl of a 20 mg/ml stock) can be added to the ethanol precipitation step to increase recovery of small amounts of DNA. We have not observed any impact of carryover glycogen on downstream transformation. Resuspend the final DNA pellet in a small volume (e.g., 4 µl) of sterile water and use 2 µl DNA in transformation of* E. coli.

8. If step 7 has not been performed, dilute the Gibson assembly reaction 1:3 with 40 µl sterile water (total volume now equals 60 µl).
9. Use 2 µl diluted Gibson assembly reaction or 2 µl resuspended DNA (if step 7 was done) to mix with 50 µl electrocompetent DH10B *E. coli* (or similar strain). High efficiency competent cells (>10^9 cfu/µg) are recommended.
10. Transfer the mixture to a 0.1 cm cuvette and pulse in an electroporation apparatus. We use 2.0 kilovolts, 200 ohms, and 25 microFarads for a Gene Pulser unit (Bio-Rad). This typically produces a time constant in the range of 4.0–4.3 msec.
11. Add 1 ml sterile SOC medium to the cuvette to recover the transformation mix and transfer to 15 ml snap-cap culture tubes (keep the lid in the "loose" position to allow aeration).
12. Incubate at 37°C for 1 hour with shaking to allow for antibiotic marker gene expression.
13. Subsequently, spread 2 µl, 20 µl, or 200 µl of the expression culture onto LB-Kanamycin (50 µg/ml) plates. Include untransformed *E. coli* as a negative control.
14. Incubate plates overnight at 37°C.
15. Screen transformants by picking colonies into LB-Kanamycin (50 µg/ml) broth, growing overnight at 37°C and preparing plasmid DNA minipreps using the method of your choice (e.g., Qiagen Spin miniprep kits).

    We commonly that find 10 transformants are sufficient to recover a correct targeting vector clone, although additional transformants may need to be screened in some cases.

16. Survey transformant DNA preps by restriction digestion.

The utility of this step depends upon the introduction or alteration of a specific restriction endonuclease site being present in the protospacer region. Otherwise, replacement of the protospacer sequence will not be detected by digestion as the size of the sgRNA cassette does not change by swapping specific protospacers. Even in the latter case, however, it is still helpful to do a restriction analysis to check against large scale recombination events. AccI is an example of an enzyme that gives a set of band sizes that can be easily assessed. In this case, intact targeting vectors produce bands of 5.2 kb, 4.5 kb, 3.9 kb and 0.8 kb. Additionally, any clones arising from carryover of undigested pPTS608-Cas9-hyg vector into the Gibson assembly will lack insert and have an altered digestion pattern. See Figure 3 for an example gel.

[*Place figure 3 near here.]

17. Analyze the protospacer regions of the transformants by Sanger sequencing using primers located at least 100 nucleotides upstream or downstream of the protospacer region. Typically, we design sequencing primers to check the integrity of the entire sgRNA cassette.
18. Once a correct targeting vector has been identified, prepare additional plasmid DNA, if needed, for use in electroporation of *Agrobacterium*.

Support Protocol 1: Choosing protospacers in the target gene

Introduction

CRISPR-associated nuclease cut sites within target genes are defined by the presence of a 20 nt protospacer sequence located directly upstream of a protospacer-adjacent motif (PAM). The PAM sequence is characteristic of each particular Cas nuclease; for *Streptococcus pyogenes* Cas9, the most commonly used choice and the enzyme we use here, the PAM consists of NGG. Note that a Cas9-sgRNA complex produces a *double-stranded* break within the protospacer sequence (typically between the 3rd and 4th base upstream of the PAM), so the protospacer may be chosen from either DNA strand of the targeted gene open reading frame—it need not be restricted to the sense strand. Many target genes will have large numbers of possible protospacer sequences and gene editing frequency can vary with both the target gene locus and the specific sgRNA used. The challenge, therefore, is how to choose the best protospacer candidates to use for cloning into the sgRNA cassette. Fortunately, there are a variety of sgRNA design algorithms to aid in the protospacer selection process (Haeussler et al., 2016; Labun et al., 2019; Bae et al., 2014; Graham and Root, 2015; Cui et al., 2018; Bradford and Perrin, 2019a). Of course, not all online sgRNA design websites cater to less commonly used model organisms, but the genomes of several dimorphic fungi are accommodated by tefor-CRISPOR, and several other sites offer a means to request that additional genomes be added. In many cases, supplementary BLAST searching against the genome of the specific dimorphic fungal model may be needed to assess the potential for off-target binding of candidate sgRNAs. Nevertheless, even in these instances, the design services can be useful to generate an initial pool of candidates based on expected cleavage efficiencies. Furthermore,

comparing output from several sgRNA design tools allows one to choose protospacer candidates that score highly across multiple algorithms, increasing confidence in the selection of a high efficiency sgRNA (Bradford and Perrin, 2019b).

A desirable sgRNA should promote high efficiency Cas9 cleavage of the target gene and have minimal potential for nonspecific (off-target) binding. Such characteristics are what the sgRNA design algorithms try to predict. In addition to having high efficiency and low off-target prediction scores, protospacer selection may be constrained by specific location considerations. In the case of gene disruption, identifying protospacers closer to the N-terminus of the target open reading frame is desirable, as resulting frameshifts are more likely to produce a non-functional protein when they occur early in the coding sequence. Selecting protospacers that target specific functional domains of the encoded protein is another strategy (this can be as effective as 5' exon choices (Doench et al., 2016)). It is safest to avoid protospacers that are close to exon splice sites to avoid the potential for exon skipping, which in some scenarios may produce a mostly complete protein even in the absence of the skipped exon when a downstream exon is in frame. Note that the mutational outcomes generated through end-joining DNA repair of CRISPR/Ca9-induced double-stranded breaks are non-random and are influenced by the sequence of the protospacer (see Background Information). Tools for predicting the most likely repair outcomes are available (for example, see inDelphi and SPROUT in Internet Resources), which can be used as additional screening criteria for the selection of candidate protospacers. So, although the researcher does not have precise control over the mutational outcome using the system described in this chapter, guide RNA selection can help to bias the desired genetic changes.

As one example of protospacer selection, we are using the tefor-CRISPOR design website (Haeussler et al., 2016) in this support protocol because it includes the genome of several *Blastomyces* and *Histoplasma* strains. (See Internet Resources for additional sgRNA design and repair outcome prediction website links.)

Materials List

Genomic DNA sequence for your target gene of interest
Tefor-CRISPOR protospacer design website (http://crispor.tefor.net)

Steps and Annotations

1. Input the genomic sequence of the coding region of your target gene of interest, choose the organism (if available) and select the type of PAM sequence appropriate for the specific nuclease being used (SpCas9). (See Figure 4 for a screenshot of the tefor-CRISPOR entry page.)
   Beware of inputting target sequences as cDNA, as protospacers that span exon boundaries will not be present in the genome!

[*Place figure 4 near here.]

2. Examine the output page (Figure 5), which will provide a representation of the PAM sites associated with all of the returned protospacer candidates showing their locations along the target gene sequence. This display can be downloaded in a variety of formats for import into sequence viewing software (e.g., Genbank, SnapGene, Geneious, etc.). Additionally, a table (sortable by header) listing the candidate protospacer and PAM sites is provided, containing scores for predicted specificity (higher means lower off-target cleavage), efficiency (higher predicts better on-target cleavage), likelihood of generating out-of-frame deletions, and the number and location of off-target sites allowing for 0–4 mismatches within the protospacer sequence.
These scores are based on the algorithms of Hsu et al. (2013) *or Doench et al.* (2016) *for specificity and Doench et al.* (2016) *or Moreno-Mateos et al.* (2015) *for efficiency. Of course, specificity and off-target site identification depend upon the availability of the genomic sequence for the target fungal species. If your organism's genome is not yet present in the tefor-CRISPOR tool, you are encouraged to contact the authors as they are continually adding new genomes. Protospacer candidates with high (80%) or low (<20%) GC content are flagged, as these can be associated with lower editing efficiency* (Tsai et al., 2015)*. The CRISPOR documentation concedes that the efficiency scores show a relatively modest correlation with empirical cleavage assays (~0.4 correlation coefficient). We further note that these efficiency score algorithms are based on data from sgRNAs expressed in cells using RNA polymerase III (U6) or in vitro T7 promoters and may not be as predictive for guide RNAs produced in the ribozyme-flanked design we employ in this chapter. We have not yet accumulated enough comparative data on sgRNA efficiency in gene editing of* Blastomyces *to be able to comprehensively evaluate the reliability of these efficiency prediction algorithms. Nevertheless, in the absence of other approaches, we prefer to select the candidates with higher predicted efficiency among those that also meet high specificity and desirable location criteria.*

[*Place figure 5 near here.]

3. Choose at least two or more candidate protospacer sequences from a given gene for incorporation into targeting vectors. Tefor-CRISPOR recommends using guide sequences with a specificity score of at least 50 and these candidates are colored in green on the sequence map. For further detailed information on the use of tefor-CRISPOR, the reader is referred to the documentation accompanying that website.

Basic Protocol 2: *Agrobacterium*-mediated Transformation of *Blastomyces*

Introduction

Once a completed targeting vector has been generated, it is necessary to introduce the vector into *Blastomyces*. This is accomplished in a two-step process where the vector is first electroporated into *Agrobacterium* and subsequently, *Agrobacterium* transformants are co-cultivated with *Blastomyces*

yeast under conditions appropriate for the induction of conjugative transfer of the targeting vector DNA to the fungal cells. Selection of *Blastomyces* transformants over a 1- to 2-week period leads to individual colonies, which can be expanded for genomic DNA isolation, PCR amplification of the targeted gene region and sequencing to determine which clones have mutations in the targeted gene. For an overview timeline to plan out the coordination of *Agrobacterium* and *Blastomyces* culturing steps, see Figure 6.

[*Place figure 6 near here.]

Materials List

Electrocompetent *Agrobacterium tumefaciens* LBA1100
Electroporation apparatus (e.g., Gene Pulser, Bio-Rad)
Electroporation cuvettes, 0.2 cm gap (Bio-Rad)
SOC medium
Luria-Bertani medium + 0.1% glucose + spectinomycin (100 µg/ml) + kanamycin (100 µg/ml) broth and plates (LBGSK)
15 ml snap-cap culture tubes
Agrobacterium Minimal Medium (AMM), add antibiotics prior to use
Kanamycin (50 mg/ml stock, use at 100 µg/ml)
Spectinomycin (50 mg/ml stock, use at 100 µg/ml)
Induction Medium (IM) broth, add acetosyringone (AS) prior to use
Acetosyringone (100 mM stock, use at 200 µM final concentration)
Spectrophotometer
Histoplasma Minimal Medium (HMM) broth and plates
Induction Medium + 200 µM acetosyringone (IM+AS) plates
Hygromycin B
3M + cefotaxime (200 µM) + hygromycin B (25 µg/ml) plates
Pall Biodyne A or Nytran S nylon membranes, cut to size and autoclaved
Phosphase-buffered saline (PBS)
MasterPure Yeast DNA purification kit (Lucigen), or similar

Steps and Annotations

Electroporation of Agrobacterium

1. Prepare targeting vector DNA at a concentration of 10–20 ng/µl in water.
2. Thaw electrocompetent *Agrobacterium tumefaciens* LBA1100 on ice.
   (see Support Protocol 2 for the preparation of electrocompetent Agrobacterium).
3. Add 20 ng targeting vector DNA (1 to 2 µl) to ~40 µl thawed *Agrobacterium*.
4. Transfer the mixture to a prechilled 0.2 cm cuvette and pulse in an electroporation apparatus (Bio-Rad Gene Pulser) at 2.5 kilovolts, 200 ohms, and 25 microFarads.

5. Add 1 ml sterile SOC medium to the cuvette to recover the transformation mix and transfer to 15 ml snap-cap culture tubes (keep the lid in the "loose" position to allow aeration).
6. Incubate at ≤28°C for 60–90 min with shaking to allow for antibiotic marker gene expression.
7. Spread 2 µl, 20 µl, or 200 µl of the expression culture onto LBGSK plates. Include untransformed *E. coli* as a negative control.
   Spectinomycin selects for maintenance of the helper Ti-plasmid present in A. tumefaciens *LBA1100 while kanamycin selects for the uptake of the targeting vector.*
8. Incubate at ≤28°C for 2 to 3 days. Individual colonies will be used in the following step.
   If desired, these plates may be wrapped in parafilm and stored at 4°C for up to 4 weeks prior to re-streaking.
   Transformation efficiency is typically on the order of 10^5 transformants per µg of plasmid DNA. Although this is lower than is common with E. coli*, it suffices for moving plasmids from* E. coli *into* Agrobacterium*.*

Pre-cocultivation Growth and Induction of Agrobacterium

9. Pick a single colony from step 8 and streak out the *Agrobacterium* transformant onto an LBGSK plate.
10. Incubate 2 to 3 days at ≤28˚C.
11. Two days before starting cocultivation, inoculate 5 ml of AMM + kanamycin (100 µg/ml) + spectinomycin (100 µg/ml) in a 50 ml sterile flask with a hefty inoculum (e.g., 10 colonies) from the restreaked plate (step 9).
    Do not use just a single colony, as it will not grow up enough overnight. Add antibiotics to AMM broth immediately before use.
12. Incubate overnight with shaking (170–250 rpm) at ≤ 28˚C.
13. The following afternoon, measure OD_{600} of the culture using a 1:5 dilution in AMM. Calculate the corrected OD_{600} to account for the dilution factor.
14. Prepare 10 ml IM broth + 200 µM acetosyringone (20 µl of a 100 mM stock) in a 50 ml sterile flask and inoculate with an appropriate volume of AMM starter culture to give a target OD_{600} = 0.05.
    Calculate the volume of starter culture to add using: corrected $OD_{600} \cdot V_1 = 0.05 \cdot (10\ ml + V_1)$ or after rearrangement, $V_1 = 0.5 / (\text{corrected } OD_{600} - 0.05)$.
15. Incubate overnight with shaking (170–250 rpm) at ≤ 28˚C.
16. The following morning, measure OD_{600} using a 1:5 dilution in IM and calculate the corrected OD_{600} to account for the dilution factor.
17. If the corrected OD_{600} is ~0.6, proceed to step 19 and use 100 µl for each cocultivation.
18. If the corrected OD_{600} is significantly greater than 0.6 (as is often the case), dilute the cultures back to a target OD_{600} =0.4 with IM+AS media and grow for ~2–2.5 hours at ≤ 28˚C and recheck the OD_{600}.
    To dilute back, remove volume from each culture to leave 5 ml remaining; add volume (V_a) of IM+AS calculated as follows:
    corrected OD_{600} $\cdot 5\ ml = 0.4\ (5\ ml + V_a)$

or after rearrangement, V_a = (*corrected* OD_{600} ·5 ml − 2) / 0.4

The goal is to have the Agrobacteria be in log phase when used for cocultivation.

19. Once the corrected OD_{600} reaches ~0.6, place the cultures on ice until preparing cocultivation mixtures.

Pre-cocultivation Growth of Blastomyces

20. Two to four days prior to cocultivation, inoculate HMM plates with the desired strain of *Blastomyces* and grow at 37˚C.

    Approximately 10^7 yeast per cocultivation will be needed and one can expect ~10^8 cells per plate so plan the number of Blastomyces cultures based upon the number of cocultivations to be done.

    For an experiment needing up to 25 cocultivation plates (i.e., 2.5 x 10^8 Blastomyces yeast cells), spread three HMM plates. This should yield ≥ 2–4 x 10^8 cells upon harvest.

21. On the day of cocultivation, harvest the slants or plates with 5 ml HMM broth. Pipet the medium onto the plate and gently dislodge the *Blastomyces* cells using a sterile loop, followed by gentle pipetting. Transfer the cell suspension into a conical tube.

    Alternatively, yeast cells can be directly removed using a sterile loop and transferred into a conical tube containing the HMM broth.

22. Count the *Blastomyces* yeast cells using a hemocytometer using a 1:10 or 1:100 dilution in 1X PBS.

    Take note on the condition of the cell suspension, examining the extent to which the cells exhibit pseudohyphal growth (short elongated cell morphology that is not true mold hyphal growth). Relatively modest amounts of pseudohyphae are not problematic but if cultures have greater than ~50% pseudohyphal cells, it may be preferable to use new Blastomyces *yeast cultures.*

    Blastomyces *yeast cells are often present in clusters and many budding daughter cells remain attached to the parent cell, characteristics that make obtaining accurate counts somewhat challenging. It is best to establish consistent counting practices as to how one chooses to count such daughter cells and clusters.*

23. Centrifuge the harvested cell suspension at 800 x *g* (2000 rpm for Beckman Allegra R with GH-3.8 rotor) for 5 min at room temperature.
24. Withdraw the supernatant and resuspend the cell pellet in an appropriate volume of HMM broth to achieve a concentration of 10^8 yeast/ml.

    The cells are now ready to use for cocultivation.

COCULTIVATION

Growth on Induction Media

25. Using sterile forceps, place autoclaved Biodyne A membranes onto IM+AS plates.

    Membrane materials affect the outcome of AMT experiments. We find that nylon membranes such as Biodyne A or Nytran S work well. Empirical testing of various types and

brands of membrane may be required to find one that supports the optimal growth of your desired fungal strain.

26. Mix 100 µl of *Agrobacterium* culture with 100 µl (10^7 cells) of *Blastomyces* suspension in a sterile microfuge tube.
    Typically, each cocultivation is replicated on 3–5 plates so these volumes are scaled to create a master mix (e.g. a 6X master mix for 5 plates would use 600 µl each of Agrobacterium *and yeast). Often, a set-up table can be helpful to organize all of the cocultivation groups in an AMT experiment, including controls. See Table 2 as an example.*
    Note that the optimum number of yeast cells may be lower than this amount and may need to be determined experimentally for each strain.

[*Place table 2 near here.]

27. Spread 200 µl of the *Agrobacterium* + *Blastomyces* mixture onto each membrane of the IM +AS plates.
    For Agrobacterium-*only or* Blastomyces-*only controls, spread 100 µl of the respective cell suspensions.*
28. Incubate at ≤ 28˚C for 2 to 3 days.

Growth on Selection Media

29. On the second or third day of cocultivation, aseptically transfer each membrane from the IM+AS plate to a 3M + cefotaxime (200 µM) + hygromycin B (25 µg/ml) selection plate.
    Cefotaxime kills off the Agrobacterium*, while hygromycin B selects for* Blastomyces *transformants. Hygromycin B potency differs among strains and drug batches so testing a variety of higher or lower concentrations may be required to find the best balance between inhibiting background growth of untransformed yeast and generating high numbers of transformant colonies.*
30. Incubate the plates at 37˚C and monitor growth of putative transformants from 1 to 3 weeks, until no additional colonies form.
    Picking colonies after 10–14 days of selection is typical.

Picking & Screening of Blastomyces Transformants

31. Once colonies have attained a size sufficient for picking (e.g., ~3 mm), streak as a small patch onto a 3M+cef+hyg selection plate marked with a grid. Multiple clones can be patch-streaked on a single plate.
32. Incubate at 37°C for several days until each patch has sufficient growth for further passaging.
33. Continue to passage onto larger sectored plates until full plate growth is attained for each clone.
34. Use a portion of the full plate growth to freeze down one or more vials per *Blastomyces* transformant clone (s*ee Support Protocol 3).*

35. Use a loopful of yeast to prepare genomic DNA using a MasterPure Yeast DNA purification kit (Lucigen) or similar.
    Follow the manufacturer's protocol with the following modification: after the yeast cells are suspended in the lysis buffer provided in the kit, vortex for 10 sec and incubate for 60 min at 60°C to lyse cells. Proceed with the kit's instructions. Note that the amount of DNA recovered using such kits may not be suitable for applications such as Southern blotting but should be sufficient for PCR analysis.
36. Screen the genomic DNA preps for mutations in the region of the target gene protospacer by amplifying the surrounding region by PCR and analyzing by Sanger sequencing.

Support Protocol 2: Preparation of electrocompetent *Agrobacterium*

Introduction

In order to introduce targeting vector DNA into *Agrobacterium*, the cells must be made "competent" to take up the plasmid DNA by electroporation. This simple procedure may be done prior to the day on which the electroporation is performed and aliquoted stocks stored frozen for a year or more, although fresh electrocompetent cells should be prepared if difficulty in transformation is experienced.

Materials List

Agrobacterium tumefaciens LBA1100
Luria-Bertani medium + 0.1% glucose + spectinomycin (100 µg/ml) broth and plates (LBGS)
1 mM Hepes, pH 7.0, filter sterilized
10% glycerol, filter sterilized
Sorvall centrifuge (or similar)
Oakridge tubes, autoclaved
Sterile 0.5 ml microfuge tubes
Liquid nitrogen or crushed dry ice mixed with ethanol

Steps and Annotations

1. Streak out *Agrobacterium tumefaciens* strain (e.g., LBA1100) on LBGS plates and grow at ≤28˚C for 2 to 3 days.
   Plates can be wrapped in parafilm and stored at 4˚C for several days until needed.
2. Use a single colony to inoculate a preculture of 2 ml LBGS broth in a 25 ml sterile flask and incubate at ≤28˚C with shaking (~250 rpm) for 6 hr.
3. Inoculate two overnight cultures of 100 ml LBGS broth each in 500 ml sterile flasks with 100 µl of preculture; incubate overnight at ≤28˚C with shaking (250 rpm).
4. The next morning, chill cultures on ice for 15 min.
   From this point on, care should be taken to maintain the bacterial cells at 4°C.

5. Transfer overnight cultures to 3 prechilled Oakridge tubes per 100 ml of culture and spin at 4000 x *g* (e.g., 5790 rpm for a SS-34 rotor) in a prechilled rotor and centrifuge at 4˚C for 20 min.
6. Discard the supernatant and resuspend the cell pellet in 10 ml per 3 tubes with prechilled 1 mM Hepes (7.0); pool 3 pellets into one tube.
7. Spin at 4000 x *g* for 20 min at 4˚C.
8. Resuspend each pellet in 10 ml in prechilled 10% glycerol and spin at 4000 x *g* for 20 min at 4˚C.
9. Resuspend each pellet in 500 µl of prechilled 10% glycerol and pool the two pellets.
10. Dispense *Agrobacterium* suspension into 40 µl aliquots in chilled sterile 0.5 ml microfuge tubes.
11. Flash-freeze the aliquoted cells in liquid N2 or a dry ice/ethanol slurry and store frozen at -80˚C.

Support Protocol 3: Preparation and recovery of *Blastomyces* frozen stocks

Introduction

Once single clones of *Blastomyces* transformants have been expanded for screening, it is important to preserve frozen stocks of early passage cultures to guard against accidental loss due to culture contamination or unexpected mishaps while awaiting results from target gene DNA sequencing. This is done by freezing cells in a cryoprotective medium (10% glycerol) at a controlled rate of cooling using isopropanol-filled freezing containers. Fungal cells are stable during long-term storage in liquid nitrogen.

Materials List

Actively growing cultures of *Blastomyces*
Sterile 2 ml cryovials
10% glycerol, autoclaved
Isopropanol-filled freezing container (e.g., “Mr. Frosty”, ThermoFisher)
Histoplasma Minimal Medium (HMM) broth and plates

Steps and Annotations

Freezing

1. Harvest *Blastomyces* clones from healthy confluent culture plates by scraping with a sterile loop and transferring into a conical tube containing 1 ml sterile 10% glycerol.
2. Mix the cell suspension well and distribute into ~200–300 µl aliquots into prelabelled cryovials.

3. Place sealed cryovials into a freezing container with the reservoir filled to the indicator line with isopropanol.
4. Place freezing containers at -70°C overnight.
5. Transfer to liquid nitrogen the following day.

Thawing and Recovery

6. To start up a culture from the frozen stock, quickly remove the cryovial from liquid nitrogen storage and temporarily place into a secondary container for transport to a biosafety cabinet.
   The secondary container provides containment in the event that a cryovial bursts from rapid expansion of liquid nitrogen vapors that may be contained within the cryovial.
7. In a biosafety cabinet, briefly loosen the cryovial lid to allow for the escape of any nitrogen vapors, and then reseal the lid.
8. Quickly thaw the contents by partially submerging the cryovial in a 37°C water bath.
   Do not submerge the entire cryovial below the water surface as this increases the opportunity for bacterial contamination of the culture if the mouth of the vial is submerged.
9. (Optional) Transfer the contents of the cryovial into a 15 ml conical tube containing 10 ml of HMM broth and spin at 800 x *g* for 10 min at room temperature. Remove the supernatant and resuspend the cell pellet in ~200 µl HMM medium.
   This step allows for the removal of the glycerol present in the freezing medium and provides an opportunity to concentrate the inoculate for plating. Generally, we have found this step to be unnecessary, but it may be useful when a frozen stock was made with a low cell density or for strains that are particularly sensitive to the stress of freezing and therefore are harder to recover.
10. Plate the entire cell suspension onto HMM plates and grow at 37°C.
    It often takes 2 or 3 passages before the fungal cultures look vigorous and healthy, such that they are ready to use for downstream applications.

Reagents and Solutions

Acetosyringone, 100 mM (500X AS)

Aldrich D13440-6 (3', 5'-dimethoxy-4'-hydroxyacetophenone)

Add 196 mg to 10 ml ethanol. Filter sterilize and store at -20°C. May need to warm to 37°C to redissolve prior to use.

Agrobacterium Minimal Medium (AMM) broth

Final		For 500 ml
0.205%	K_2HPO_4	1.025 g

0.145%	KH_2PO_4	0.725 g
0.015%	NaCl	75 mg
2 mM	$MgSO_4$ (anhydrous)	0.12 g
0.45 mM	$CaCl_2 \cdot 2H_2O$	33.5 mg
9 µM	1 mM $FeSO_4 7H_2O$	4.5 ml
0.05%	$(NH_4)_2SO_4$	0.25 g
0.20%	Glucose	1.0 g
	Adjust to pH ~ 6.8	
	H_2O to final volume	500 ml

Filter sterilize.

Histoplasma Minimal Medium (HMM) broth

To approximately 800ml of H_2O, add:
10.6 g Ham's F-12 powder
1.0 g glutamic acid (monosodium salt)
18.2 g glucose (dextrose)
5.96 g HEPES
10 ml 100X L-cystine
Adjust to pH 7.5 (*the solution should be reddish-pink in color*)
Bring volume to 1L with H_2O.
Filter sterilize and store at 4°C.

Histoplasma Minimal Medium (HMM) plates

HMM plates are prepared as two components. This makes 1 liter.

Part A: 2X agarose

Add 5 g SeaKem LE agarose to 480 ml water in a 2L flask and autoclave. Cool to 56°C.

Part B: 2X broth

Start with 400 ml water and add the following ingredients:

10.6 g Ham's F-12 powder
1.0 g glutamic acid (monosodium salt)
18.2 g glucose (dextrose)
5.96 g HEPES
10 ml 100X L-cystine
Adjust to pH 7.5

Bring volume to 500 ml with H_2O.
Filter sterilize

Part C: mixing and additives
Combine:
Part A (480 ml)
Part B (500 ml)
10 ml sterile 1 mM $FeSO_4 \cdot 7H_2O$ (100X)
10 ml penicillin (10,000 unit/ml)-streptomycin (10 mg/ml) solution (100X)

Hygromycin B, 100 mg/ml
A.G, Scientific (#H-1012)
Stable at 4°C for 2 years.

Induction Medium (IM) broth

Final		For 500 ml
0.205%	K_2HPO_4	1.025 g
0.145%	KH_2PO_4	0.725 g
0.015%	NaCl	75 mg
2 mM	$MgSO_4 \cdot$ (anhydrous)	0.12 g
0.45 mM	$CaCl_2 \cdot 2H_2O$	33.5 mg
9 µM	1 mM $FeSO_4 \cdot 7H_2O$	4.5 ml
0.05%	$(NH_4)_2SO_4$	0.25 g
40 mM	MES (Sigma M2933)*	3.9 g
10 mM	1 M glucose	5 ml
0.5%	100% glycerol	2.5 g
	Adjust to pH 5.3	
	H_2O to final volume	500 ml

*MES hydrate, 4-morpholineethanesulfonic acid

Filter sterilize. Prior to use, add 100 mM acetosyringone to a final concentration of 200 uM.

Induction Medium (IM+AS) plates
Induction medium plates are prepared as two components. This recipe makes 1 liter.

Part A: 2X agarose

Add 5 g SeaKem LE agarose to 500 ml water in a 2L flask and autoclave. Cool to 56°C.

Part B: 2X broth

Start with 400 ml water, add the following ingredients and bring the volume to 500 ml with additional water.

Final conc.		2x
0.205%	K_2HPO_4	2.05 g
0.145%	KH_2PO_4	1.45 g
0.015%	NaCl	0.15 g
2 mM	$MgSO_4$ (anhydrous)	0.24 g
0.45 mM	$CaCl_2 \cdot 2H_2O$	67 mg
9 µM	1 mM $FeSO_4 \cdot 7H_2O$	9 ml
0.05%	$(NH_4)_2SO_4$	0.5 g
40 mM	MES (Sigma M-2933)	7.8 g
5 mM	1 M glucose	5 ml
0.5%	100% glycerol	5 g
200 µM	100 mM Acetosyringone	2 ml
	adjust pH to 5.3 with HCl	
	H_2O to final volume	500 ml

Filter sterilize and warm to 56°C.

Mix Part A & Part B and pour into plates. 1L makes about 40 plates.

Luria-Bertani (LB) medium + 100 µg/ml kanamycin broth & plates

For 1L, to 900 ml H_2O, add:
5 g yeast extract
10 g tryptone
10 g NaCl
Adjust to pH 7.0 and bring volume to 1L with H_2O.
Autoclave and store at 4°C for 6 months.
Add 50 mg/ml kanamycin (500X) to final concentration of 100 µg/ml prior to use.

For plates, include 15 g agar prior to autoclaving. Cool to 56°C, add 2 ml kanamycin (50 µg/ml stock) and pour plates. Store at 4°C for 6 months.

Luria-Bertani medium + 0.1% glucose + spectinomycin ± kanamycin (LBGS or LBGSK) broth & plates
For 1L, to 900 ml H_2O, add:
5 g yeast extract
10 g tryptone
10 g NaCl
Adjust to pH 7.0 and bring volume to 995 mL with H_2O.
Autoclave and cool.
Add 5 ml sterile 20% glucose.
Store at 4°C for 6 months.
Add 50 mg/ml spectinomycin (500X) and 50 mg/ml kanamycin (500X), if appropriate, to final concentration of 100 µg/ml each prior to use.

For plates, include 15 g agar prior to autoclaving. Cool to 56°C, add 2 ml spectinomycin (50 µg/ml stock) and 2 ml kanamycin (50 µg/ml stock), if appropriate, and pour plates. Store at 4°C for 6 months.

SOC medium
For 1L, add to 900 ml H_2O:
20 g tryptone
5 g yeast extract
0.5 g NaCl
Once dissolved, add 10 ml 250 mM KCl.
Adjust to pH 7.0 and bring volume to 1L with H_2O.
Autoclave for 20 min and cool to 56°C
Add 20 ml filter sterilized 1 M glucose.
Store at 4°C for 6 months.

3M+cef+hyg selection plates
3M selection medium is prepared in three parts. This makes 1L.

Part A: 2X agarose
Add 0.5 g SeaKem LE agarose to 465 ml water in a 2L flask and autoclave. Cool to 56°C.

Part B: 2X broth

glucose	10 g
K_2HPO_4 (anhydrous)	1.5 g
$(NH_4)_2SO_4$	2 g
L-proline	2 g

100X L-cystine	0.2 ml
adjust to pH 7.0	
H_2O to final volume	500 ml

Filter sterilize and warm to 56°C.

Part C: mixing and additives

Part A: 2X agarose	465 ml
Part B: 2X broth	500 ml
1 mM Fe $SO_4 \cdot 7H_2O$	10 ml
penicillin (10,000 unit/ml)-streptomycin (10 mg/ml) solution (100X)	10 ml
2M $MgSO_4$ (1000X)	1 ml
0.2 M $CaCl_2$ (1000X)	1 ml
3M vitamin mix (1000X)	1 ml
3M trace elements (100X)	10 ml
200 mM cefotaxime	1 ml
Hygromycin B (100 mg/ml)	250 µl
Final volume	1000 ml

3M Stock Solutions

0.2 M Calcium Chloride (1000X)

For 100ml:

Mix 3.0 g $CaCl_2 \cdot 2H_2O$ in 80 ml H_2O.

Bring volume to 100 ml with H_2O and autoclave.

Store at room temperature.

100X L-Cystine

Mix 840 mg L-cystine in 50 ml 0.5 M HCl.

Bring volume to 100 ml with H_2O and filter sterilize.

Store at room temperature.

2M Magnesium Sulfate (1000X)

For 100ml:

Mix 50.0 g $MgSO_4 \cdot 7H_2O$ in 70 ml H_2O.

Bring volume to 100 ml with H_2O and autoclave.

Store at room temperature.

Trace Elements stock

For 500 ml, add the following to 450 ml H_2O:

28.5 mg boric acid (H_3BO_3)

50 mg cupric sulfate ($CuSO_4$)

26 mg manganese sulfate heptahydrate ($MnSO_4 \cdot 7H_2O$) (or alternatively, 15.86 mg $MnSO_4 \bullet H_2O$)

18 mg ammonium molybdate tetrahydrate [$(NH_4)_6Mo_7O_{24} \cdot 4H_2O$]

396 mg zinc sulfate heptahydrate ($ZnSO_4 \cdot 7H_2O$)

970 mg ferrous sulfate heptahydrate ($FeSO_4 \cdot 7H_2O$)

Bring volume to 500 ml with H_2O.

Filter sterilize and store at 4°C.

Vitamin stock

For 100ml, add the following to 80 ml H_2O:

10 mg D-biotin

60 mg calcium pantothenate (D-pantothenic acid)

10 mg inositol (myo-inositol)

60 mg nicotinic acid

60 mg thiamine HCl (thiamine monophosphate chloride)

Bring volume to 100 ml with H_2O.

Filter sterilize and store at 4°C.

Commentary

Background Information

CRISPR/Cas technology is derived from bacterial immune systems evolved to defend against invading viral nucleic acids and plasmid DNAs (Horvath and Barrangou, 2010; Hsu et al., 2014). In its original bacterial host setting, short spacer fragments of bacteriophage DNA are incorporated into repeat regions of the bacterial genome. This feature has given these systems their moniker: “Clustered regularly interspaced short palindromic repeats.” Transcription and processing of these repeat regions produces short RNA sequences that complex with Cas nucleases to bind and degrade phage DNA during subsequent encounters. Two classes of short RNA—an invariant “tracr” RNA and the viral-derived “crispr” RNA— interact to form a scaffold for complexing with a Cas nuclease; the binding of a 20 nt protospacer sequence within the “crispr” RNA component to its complementary sequence in the invading viral genome directs the nuclease complex to its target for double-stranded cleavage. With its development as a biotechnology tool, the tracr and crispr RNAs have been combined into a “single guide RNA” (sgRNA) that incorporates both the invariant structural and target-specific protospacer functions.

A variety of Cas nucleases are present in CRISPR systems found across multiple bacterial genera and in addition to differences in protein size, protospacer length, and guide RNA structure, each Cas nuclease variety has its associated PAM sequence preferences. In the case of *Streptococcus pyogenes* Cas9, NGG located immediately 3' of the protospacer is the predominant PAM, although off-target sites using NGA and NAG have been observed (Tsai et al., 2015) and engineered forms of Cas9 may utilize variant PAMs such as NGA, NGCG or NAA among others (Kleinstiver et al., 2015; Hu et al., 2018; Nishimasu et al., 2018; also see discussion in Anzalone et al., 2020). The relatively relaxed PAM sequence of *S. pyogenes* Cas9 is advantageous on the one hand as it increases the likelihood of finding appropriate targeting sites within most genes of interest, except perhaps for the heaviest of AT-rich loci; the trade-off is that this increases the potential for off-site targeting, relative to Cas nucleases with longer, more stringent PAM requirements.

Fidelity in CRISPR targeting is dependent upon the tolerance of the Cas9 nuclease for mismatches between the sgRNA and target gene protospacer. Recognition of the target site begins with binding of the sgRNA/Cas9 complex to the PAM sequence, followed by unwinding of the target DNA and sgRNA binding to its complement, creating a DNA/RNA heteroduplex and a single stranded "R-loop" (Jiang and Doudna, 2017). The target is unwound starting closest to the PAM and mismatches in the most PAM-proximal positions are least tolerated (Sternberg et al., 2014; Wu et al., 2014; Graf et al., 2019). Once the R-loop is formed, Cas9 nuclease domains become active through conformational changes; mismatches interfere with these conformational changes and this is the basis for limiting cleavage to sites with high similarity to the guide sequence. SpCas9 nuclease generates double-stranded DNA breaks through the enzymatic action of two distinct nuclease domains (RuvC and HNH), the former cleaving the single stranded R-loop and the latter cutting within the heteroduplex (Jinek et al., 2012).

As one means to counteract the possible confounding effects of off-site targeting, we recommend employing multiple sgRNA sequences for each gene target of interest. The array of potential off-target binding sites will be specific to each guide sequence, such that one would not necessarily expect off-target sites to be shared across multiple protospacers. Phenotypic consequences of target gene disruption that are shared by clones derived from independent sgRNAs are therefore not likely to be attributable to unintended off-target effects. Perhaps future modifications to the system, such as the use of higher fidelity engineered Cas9 variants, may also lower the propensity for off-target complications. Similarly, transient expression of the CRISPR/Cas9 components has been associated with lower off-target outcomes so using an inducible promoter to drive short term Cas9 expression may be a desirable modification (Davis et al., 2015).

In addition to fidelity, the specific sequence of the guide RNA is also associated with varying levels of editing efficiency. Characteristics such as length, GC content (>50%), presence of purines near the end of the protospacer, and RNA secondary structure considerations have all been described as contributing factors in accounting for such differences (Doench et al., 2014; Wang et al., 2014; Wong et al., 2015). For example, Doench et al. (2014) found strong nucleotide preferences at specific positions of the protospacer, such as: position 3 should not contain cytosine, position 16 should be cytosine but not guanine, whereas guanine (not cytosine) is preferred at position 20. Fortunately,

such considerations have been incorporated into sgRNA design tools so that the researcher need not manually balance all of the fine details of optimal protospacer efficiency. We note, however, that efficiency scoring of protospacer candidates is not fool-proof, again underscoring the importance of selecting multiple sgRNA candidates for experimental use.

Repairing Cas9-generated double-stranded breaks may occur via several cellular pathways. Most commonly, end-joining repair produces small indels. This can proceed through classical nonhomologous end-joining (c-NHEJ) or by microhomology-mediated end joining (MMEJ or alt-NHEJ). The former is often associated with single base insertions, whereas deletions tend to be associated with the latter. In budding yeast, efficient NHEJ repair of CRISPR/Cas9-induced breaks requires DNA polymerase Pol4, Ku heterodimer, DNA ligase 4 and also depends on the Mre11-Rad50-Xrs2 complex (Lemos et al., 2018).

Error-free end joining perfectly repairs the cut site restoring the protospacer and adjacent PAM target but this intact sequence is a substrate for further rounds of CRISPR/Cas9 targeting and repair. Error-prone repair producing indels precludes subsequent recutting as the perfect match between guide RNA and target is disrupted. Although the specific bases inserted or deleted at any given target site cannot be controlled by the investigator in this scenario, indel formation is not random (see also Understanding Results). Multiple large-scale analyses have revealed that protospacer and nearby target gene sequences have a strong impact on the observed mutational spectra (van Overbeek et al., 2016; Shen et al., 2018; Allen et al., 2019; Chen et al., 2019; Leenay et al., 2019). Single base insertions are significantly biased by the specific base located immediately upstream of the cleavage site, preferentially duplicating that nucleotide (Lemos et al., 2018; Chen et al., 2019; Shen et al., 2018). Although Cas9 has traditionally been considered to generate blunt ends (Jinek et al., 2012), more recent work (Shou et al., 2018; Zuo and Liu, 2016; Gisler et al., 2019) has indicated that 1 nt 5′ overhangs can also be produced with the RuvC domain cleaving the non-template strand 4 bases upstream of the PAM (-4) and the HNH domain cutting the template strand at -3 bases relative to the PAM (Figure 7A). Fill-in of the overhanging base by DNA polymerase Pol4 during c-NHEJ provides an mechanism to account for the propensity of such duplicated nucleotide insertions (Lemos et al., 2018). As Pol4 lacks proof-reading activity, error-prone fill-in might explain the minority of cases of non-templated single base insertions. Deletions are frequently associated with short regions of microhomology (2–20 bp) in and near the protospacer. Limited resection of the DNA ends at the DNA break allows small regions of homology on each side of the break to pair, displacing the nonhomologous portions of the single stranded ends (Lemos et al., 2018). Trimming of the displaced ends and fill-in of the resulting gaps results in a corresponding deletion (Figure 7B).

An additional repair pathway, homology-directed repair (HDR), can be utilized in the presence of a donor DNA template to specify the targeted changes to be introduced into the gene of interest (Lin et al., 2014). This repair process is usually less efficient than NHEJ and is typically restricted to dividing cells. Donor DNA templates are commonly provided as single-stranded or double-stranded oligonucleotides, although this varies by application. Currently, limitations on facile introduction of short DNA fragments into *Blastomyces* make HDR impractical for CRISPR targeting.

[*Place figure 7 near here.]

Critical Parameters

High efficiency electrocompetent E. coli for transformations with Gibson assembly mix

Construction of new CRISPR targeting vectors through the assembly of insert fragments with vector backbone is often a straight-forward process but benefits from the use of high efficiency competent cells such as those available from a variety of commercial suppliers. We find that "home-made" competent cells prepared in the lab may have 1–2 orders of magnitude lower efficiency than commercially prepared cells (the latter can be up to $\geq 10^{10}$ cfu/µg DNA). In some cases, lab-prepared cells may work fine but if difficulty in cloning is experienced, this enhanced transformation efficiency may make the difference between recovery of correct targeting vector clones or not.

Agrobacterium growth at ≤28°C

The success of AMT is dependent upon the excision of the recombinant T-DNA region from the CRISPR targeting vector and this excision requires the action of many gene products supplied by the helper Ti-plasmid in the *A. tumefaciens* LBA100 strain. There is a tendency for *Agrobacterium* to lose the Ti-plasmid during extended growth above 28°C. Therefore, it is critical to maintain *Agrobacterium* cultures below this temperature threshold. We typically aim for 1-2°C below 28°C to provide a margin of safety and therefore room temperature growth is often feasible. Depending upon the geographical location and ambient temperature within the lab, however, a controlled temperature incubator may be required.

Nylon membranes

Membranes can be made from a variety of materials (such as nitrocellulose, nylon, polyvinylidene fluoride, polycarbonate) but not all materials support the growth of the dimorphic fungi equally well. Empirical testing in our hands has shown that nylon outperforms these other materials in AMT of *Blastomyces*, producing the highest numbers of colonies. This may not hold true for all dimorphic fungi, so testing the growth of your fungal strain on several membranes prior to attempting AMT for the first time is recommended. Note that even within a given material category, there may be differences in products across brands. Additionally, it may be necessary to manually cut the membranes to appropriately sized discs, if the desired membrane product is not available in a pre-cut format.

Titration of hygromycin B

The effective concentration of hygromycin B varies with each commercial batch purchased. When purchased as a powder, this is may be indicted as a specific activity on the label (for example, 920 µg active per 1 mg). Sensitivity to hygromycin B may be fungal strain dependent, as well. For these reasons, titration of hygromycin B concentration in the 3M selection plates is suggested. Realize also that periodic adjustment of the optimal concentration used may be required whenever new stock is purchased.

Proof-reading polymerase during Blastomyces gene screening steps

It is important to minimize the generation of point mutations and insertions/deletions during amplification of the targeted gene locus while screening *Blastomyces* transformants, as their presence could be mistaken for evidence of gene editing. Use proof-reading thermostable DNA polymerases (such as Phusion or Q5, among others) to reduce the occurrence of PCR-induced artefacts. Mutations that occur in unexpected locations in the screening PCR fragments (i.e., far from the expected Cas9 cut site within the protospacer) should be considered as potential PCR errors and confirmed by independent re-amplification and sequencing of the targeted gene region from these clones. As an added benefit, we have also found proof-reading polymerases to perform better on "difficult" templates compared to standard Taq polymerase.

Troubleshooting

No Fragment A or B PCR products are produced

- Vary the amount of template DNA in the PCR reaction.
- Increase the number of cycles in the PCR.
- Use an annealing temperature gradient to optimize the binding of the PCR primers.
- Use the "enhancer" reagent or alternate buffer intended for high GC or other difficult templates which is often included with thermostable DNA polymerases.
- Adjust the $MgCl_2$ concentration in the reaction.
- Try a different thermostable DNA polymerase.

No E. coli colonies are recovered after transformation with Gibson assembly mix

- Use the positive control fragment mix provided with the Gibson assembly reagent to test the enzymatic activity of the Gibson master mix. Include an intact positive control plasmid in the electroporations of *E. coli* to discern between failures of assembly versus transformation.
- Increase the amount of insert DNA fragments and vector backbone in the Gibson assembly mix.
- Vary the ratio of insert fragments to vector backbone in the assembly reaction.

E. coli transformants carry incorrect or rearranged plasmids

On occasion, screening *E. coli* transformants during target vector construction reveals incorrect clones carrying insert fragments that are larger or smaller than expected. This may particularly be the case when generating multiplexed targeting vectors as the repetition of the ribozyme and invariant portions of the sgRNA sequences shared between multiple sgRNA cassettes increases the potential for rearrangement. One may try several strategies in order to overcome this.

- Transform the Gibson assembly mix into a strain of *E. coli* that is optimized for reduced recombination frequency (e.g. ElectroMax Stbl4 cells, Life Technologies or NEB Stable cells, New England Biolabs).

- Simplify the assembly reaction by utilizing a synthetic sgRNA cassette (e.g., order as a gBlock from Integrated DNA Technologies) instead of generating multiple overlapping PCR fragments.

No Blastomyces colonies are recovered after Agrobacterium-mediated transformation

Even established labs with a successful history of employing AMT may find that some experiments produce no colonies after 3 or more weeks of selection. The following steps may be helpful in troubleshooting such situations.

- Use a preparation of *Blastomyces* that has recently been recovered from infected mouse lungs. We have found that this approach can overcome the lack of transformants that we had experienced in several experiments using older frozen fungal stocks. “Passaging through mice” would therefore be one of the earliest troubleshooting steps that we recommend.
- Prepare fresh media and associated components. This includes purchasing new acetosyringone to promote *vir* gene induction in *Agrobacterium*.
- Test several lower concentrations of hygromycin B in the 3M selection media.
- Alter the ratio of *Agrobacterium* to *Blastomyces* in the cocultivation mixes.
- Increase or decrease the growth time of the cocultivation induction plates prior to transferring to 3M selection media.
- Try a different lot number or type of membrane.
- Include a pre-existing binary vector (e.g., pPTS608-Cas9-hyg) as a control to distinguish whether the failure to generate transformants lies with newly constructed targeting vectors.
- Sequence the full recombinant T-DNA region of the targeting vectors to determine if cloning induced errors in any of the functional elements may be present.
- Check for the maintenance of the Ti-plasmid by comparing growth of *Agrobacterium* on LBG plates ± spectinomycin. A spectinomycin-sensitive bacterial strain of some sort should be included as a control for spectinomycin efficacy. Additionally, PCR amplification of portions of the *vir* gene region could be performed on *Agrobacterium* genomic DNA preps (the Ti-plasmid will copurify) to provide evidence of Ti-plasmid retention.
- Alternate media types for pre-cocultivation growth of *Blastomyces* or for selection of *Blastomyces* transformants may be considered.

Blastomyces transformants do not show targeted mutation of the gene of interest.

- Increase the number of transformants screened in order to find gene-edited clones.
- Redesign the targeting vector to use different protospacers and compare multiple targeting vectors for editing frequencies.

Understanding Results

Typical CRISPR editing outcomes

As is true in most model systems, the most frequent alterations produced by CRISPR/Cas9 gene editing in *Blastomyces* are small insertions or deletions (indels) occurring about 3 nucleotides upstream of the PAM sequence. Single base indels predominate but 2–12 bp indels have also been observed. The specific nucleotide inserted does not appear to be wholly random, but is influenced by the sequence immediately preceding the nuclease cut site. For example, among 134 edited clones that we previously characterized across 7 protospacers (Kujoth et al., 2018), 86 (64%) contained single nucleotide insertions matching the specific nucleotide present 4 bp upstream of the PAM. As discussed above, this bias from end-joining repair is consistent with the CRISPR outcomes characterized in multiple large surveys (van Overbeek et al., 2016; Shen et al., 2018; Allen et al., 2019; Chen et al., 2019; Leenay et al., 2019). The overwhelming majority of indel events that we have observed produce frameshifts in the target gene open reading frame, resulting in early termination. To date, we have generated only two clones possessing indels (3 nucleotide and 12 nucleotide deletions) that would render an in-frame protein coding sequence change.

The mutational frequency varies by targeted gene locus and by the specific protospacer sequence chosen. We have observed frequencies ranging from 4%–87% of clones screened. Not all edited clones recovered will be usable for downstream phenotyping, however. Somewhat peculiarly in *Blastomyces*, we have routinely found that about half of the transformants showing evidence of gene editing also display overlapping sequence peaks beginning at the expected site of Cas9 cleavage. These mixed peak clones can sometimes be manually deconvoluted to reveal both wild type and mutant target gene sequences. We have also observed cases where deconvolution identifies two different mutations but no wild type gene sequence. *Blastomyces* cells are multinucleated so we interpret these findings to indicate editing of only a subset of nuclei within such clones. It is therefore important to screen enough transformants to increase the likelihood of identifying "clean" mutant strains for further characterization of your biological system of interest. We generally screen 30–45 clones per sgRNA targeted, although fewer or more may be appropriate for any particular protospacer. Multiplexed targeting will require screening more transformants than single gene targeting unless both target genes happen to be editable at similarly high frequencies. We have not noticed any significant change in editing frequency for a given protospacer whether in the context of single or dual gene targeting. Rather, one protospacer in a multiplex targeting often ends up producing a lower targeting frequency than the other protospacer with which it is paired and this becomes the determining factor on how many clones need to be screened, especially in light of the heterokaryon phenomenon discussed above.

Future expansion of applications

So far, we have employed CRISPR/Cas9 editing solely to produce gene disruption events. Numerous creative adaptations of CRISPR technology have been devised for use in other model systems and these include modifications to allow for targeted transcriptional activation or repression, larger scale chromosomal region deletions, epigenetic editing, as well as live imaging and chromatin

immunoprecipitation applications to name a few examples (Hsu et al., 2014; Dominguez et al., 2016). Some of these approaches should be feasible in the dimorphic fungi as well. For example, CRISPR-based transcriptional regulation fuses a transcriptional regulatory domain to a catalytically inactive "dead" (dCas) protein, which is directed to the desired target gene through complexing with the appropriate protospacer (Qi et al., 2013). The system described in this chapter could in theory be easily adapted to such an approach by replacing the Cas9 in the binary targeting vector with a suitable dCas/transcriptional regulatory domain fusion protein.

Another common goal of CRISPR/Cas9 gene editing is the introduction of specific sequence changes into the target gene, rather than gene inactivation via frameshift and premature termination. This approach requires the provision of a homology-directed repair template to dictate the desired changes, usually in the form of an oligonucleotide co-transfected along with the targeting vector. This approach is not likely to be practical due to the relative inefficiency of electroporation in the dimorphic fungi. If a repair template could be simultaneously introduced, perhaps as an additional component of the targeting vector that is "liberated" by CRISPR/Cas9 cleavage of flanking sequences, it may become possible to gain an additional level of control over gene editing in these fungal models. It remains to be seen whether homologous recombination frequencies would be sufficient to recover HDR-repaired clones among other NHEJ-generated edited transformants in such a scenario. As alluded to earlier in Background Information, many other applications of CRISPR technology have been devised and await potential adaptation for use in the dimorphic fungi.

Time Considerations

The following are estimates of the time involved in the construction of CRISPR/Cas9 targeting vectors:

- Preparation of vector backbone (4–5 hr) and PCR amplification of insert fragments (3–4 hr), including agarose gel evaluation.
- Gibson assembly and *E. coli* transformation (3–4 hr, followed by overnight growth for bacterial colonies).
- Screening *E. coli* transformants for correct targeting constructs requires overnight growth of cultures, plasmid DNA minipreps (1–2 hr, varies with number of transformants), restriction analysis (4–5 hr), Sanger sequencing and clean up (~5 hr).

For a timeline overview of *Agrobacterium* and *Blastomyces* culturing events in AMT, see Figure 6. Estimates for some individual stages include:

- *Agrobacterium* electroporation (~2 hr, followed by overnight growth of bacterial colonies).
- Harvest and preparation of *Agrobacterium* for cocultivation (~3 hr)
- *Blastomyces* harvest and cocultivation mix preparation (1–2 hr)
- Post-AMT selection of *Blastomyces* transformants (12–21 days), picking clones and passaging until freezing (~2–3 weeks)

- Screening *Blastomyces* clones for targeting requires genomic DNA preps (3–4 hr), target gene amplification by PCR, gel evaluation and post-PCR clean-up (5–6 hr), followed by Sanger sequencing and cleanup (~5–6 hr).

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Conflicts of Interest

The authors declare that they have no conflicts of interest.

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Key References with Annotations

Nødvig, C. S., Nielsen, J. B., Kogle, M. E., and Mortensen, U. H. 2015. A CRISPR-Cas9 System for Genetic Engineering of Filamentous Fungi. *PLoS ONE* 10:e0133085.

Describes the ribozyme-flanked sgRNA and Aspergillus-optimized Cas9 expression cassettes that were subseqeuntly modified by us for use in the dimorphic fungi.

Sullivan, T. D., Rooney, P. J., and Klein, B. S. 2002. Agrobacterium tumefaciens Integrates Transfer DNA into Single Chromosomal Sites of Dimorphic Fungi and Yields Homokaryotic Progeny from Multinucleate Yeast. *Eukaryotic cell* 1:895–905.

Describes the development of Agrobacterium-mediated transformation as used with dimorphic fungi and characterizes the outcome events generated by this system.

Internet Resources with Annotations

http://nebuilder.neb.com

NEBuilder Assembly Tool: Helpful for designing primers for Gibson Assembly cloning reactions.

http://crispor.tefor.net

Tefor CRISPOR: Design tool for selecting protospacer sequences located within target genes of interest; allows screening for off-target sites in Blastomyces *and* Histoplasma*.*

https://chopchop.cbu.uib.no

CHOPCHOP v3: Design tool for selecting protospacers but does not currently have dimorphic fungal genomes included so subsequent screening of candidate protospacers should be done using NCBI BLAST.

http://www.rgenome.net/cas-designer/

Cas-Designer: Design tool for protospacer selection. It includes fungal genomes but not currently those of dimorphic fungi.

https://www.crisprindelphi.design

inDelphi: CRISPR mutation prediction tool for predicting the frameshift frequency of candidate sgRNAs. This machine learning model was trained on data from human cells. The relative frequency of 1 bp insertions to deletions may vary among different cell types.

https://zou-group.github.io/SPROUT

SPROUT: Prediction tool for finding most likely repair outcomes of a given sgRNAs (e.g., most likely insertion). Based upon data from primary human T cells.

Figure Legends

Figure 1. **Gene targeting vector design. (A)** Cas9-only control (left) or sgRNA-containing targeting vector (right) maps. **(B)** Primary sgRNA transcripts containing flanking ribozyme sequences are self-excised to generate functional sgRNAs that direct Cas9 cleavage to complementary sites in the genome. Note that the hammerhead (HH) ribozyme forms an inverted repeat with the 5' end of the target-specific protospacer sequence. **(C)** Targeting vectors contain single or dual sgRNAs to target gene loci, a fungal codon-optimized *Cas9* gene (Nødvig et al., 2015), hygromycin resistance marker, and border repeat sequences (RB, LB) necessary for *Agrobacterium*-mediated gene transfer into

Blastomyces. (i) Some constructs contain only a single target sgRNA cassette. (ii) In dual targeting constructs, sgRNAs are each embedded within flanking ribozyme sequences, are separated by a random linker (hatched bar), and share a single promoter and terminator region driving their expression. PgpdA, *A. nidulans gpdA* promoter; HH, hammerhead ribozyme; IR, inverted repeat forming region of the HH ribozyme; GOI, gene of interest; HDV, hepatitis delta virus ribozyme; TtrpC, *A. nidulans trpC* terminator; Ptef1, *A. nidulans tef1* promoter; Cas9, *Streptococcus pyogenes* CRISPR-associated nuclease 9, codon-optimized for *Aspergillus*; NLS, simian virus 40 large T antigen nuclear localization signal, appended to the C-terminus of Cas9; Ttef, *A. nidulans tef1* transcriptional terminator; PtrpC, *A. nidulans trpC* promoter; hph, hygromycin B phosphotransferase; LB, RB, *Agrobacterium* T-DNA left or right border repeat, respectively; KanR, kanamycin-resistance (aminoglycoside 3′ phosphotransferase) gene; pBR325 ori, origin of replication in *E. coli*; ori REP, origin of replication from pVS1 (Itoh et al., 1984); ori STA, replication stability region from pVS1.

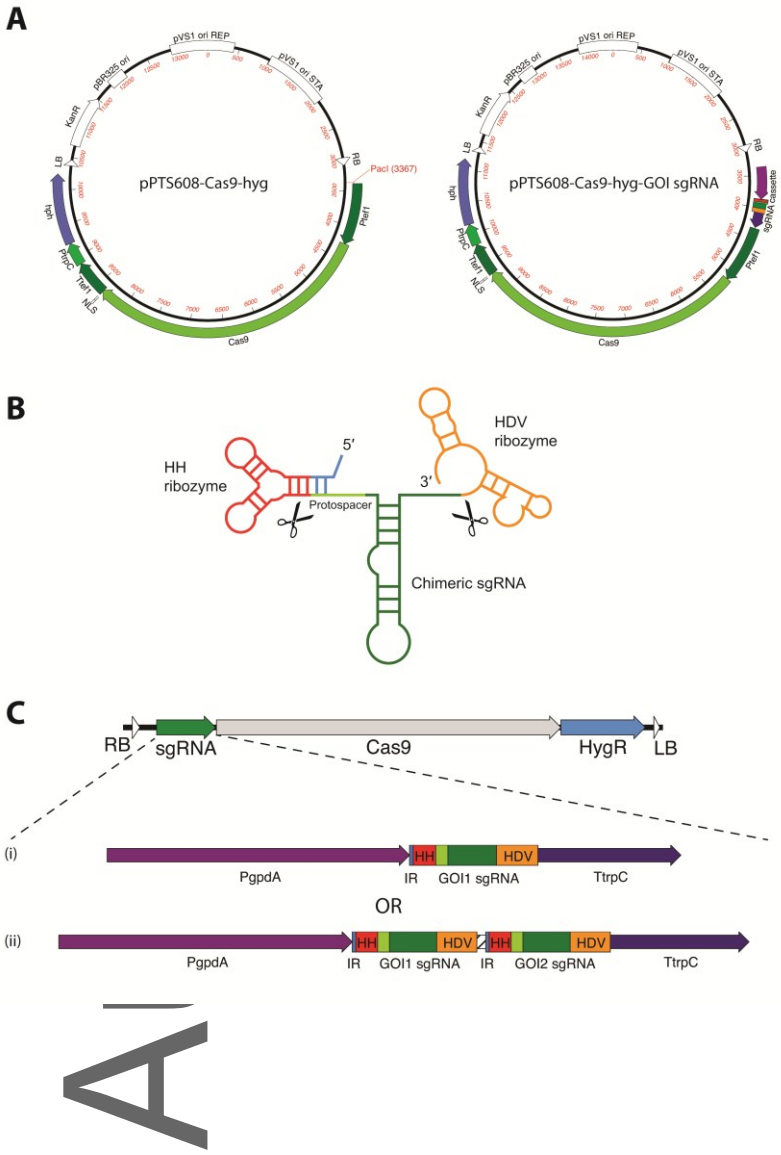


Figure 2. **Detailed cloning diagram for CRISPR sgRNA cassette construction**. **(A)** Replacement of gene-specific protospacers in the CRISPR/Cas9 targeting vectors is done by generating two overlapping fragments, A and B, that create the entire promoter-sgRNA-terminator cassette, which is then inserted into the vector backbone (not shown, but see Figure 1A). Target gene-specific sequences are included in the overlapping inner primers (FragA R and FragB F). **(B)** A detailed view of the overlapping inner primer region illustrates that the 20-nucleotide, gene-specific protospacer sequence (here, GCTGGGTTCCAATCTGCATG) is included in the Fragment B forward primer. Because the 5′ end of the hammerhead ribozyme forms an inverted repeat with the first 6 bases of the protospacer (HH IR), the corresponding 6 bases in the 5′ hammerhead region must also be replaced with gene-specific sequence (blue box, GCTGGG) in the Fragment A reverse primer. Note that although the 6 bases replaced in both primers are identical, they are on opposite strands and will form complementary repeats in the primary transcript. Abbreviations are as given in Figure 1 legend.

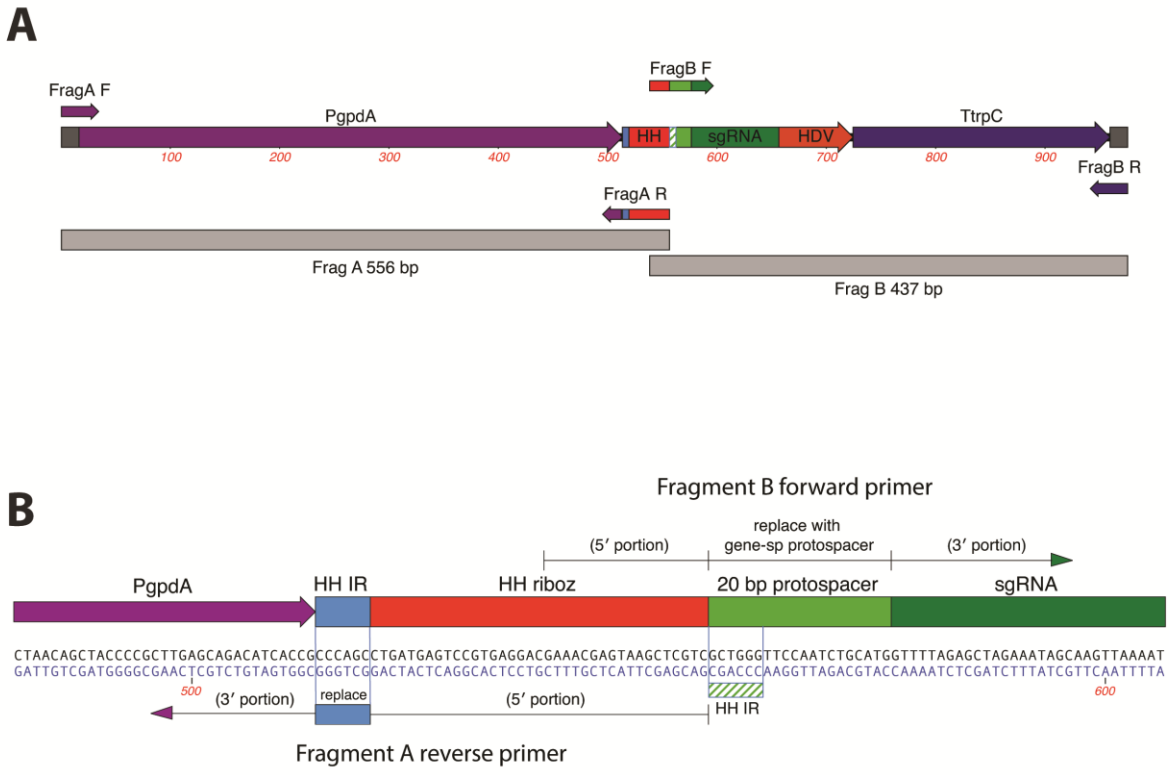


Figure 3. **AccI digestion of CRISPR targeting vector candidates**. Agarose gel (0.8%) electrophoresis of plasmid DNA minipreps from pPTS608-Cas9-hyg-GOI sgRNA candidate *E. coli* transformants digested with AccI for 4 hr at 37°C. Note presence of diagnostic bands of 5.2 kb, 4.5 kb, 3.9 kb and 0.8 kb in correct clones (C) versus unexpected band sizes in incorrect (I) clones. The size of relevant molecular weight DNA ladder bands (MW) are indicated in kilobases (kb). Note that replacment protospacer sequences in the correct clones must be confirmed by Sanger sequencing.

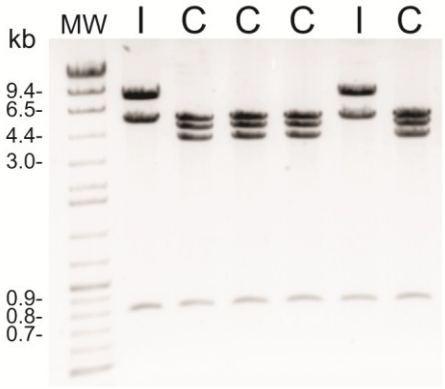


Figure 4. Screenshot of the entry page of the tefor CRISPOR sgRNA design tool.

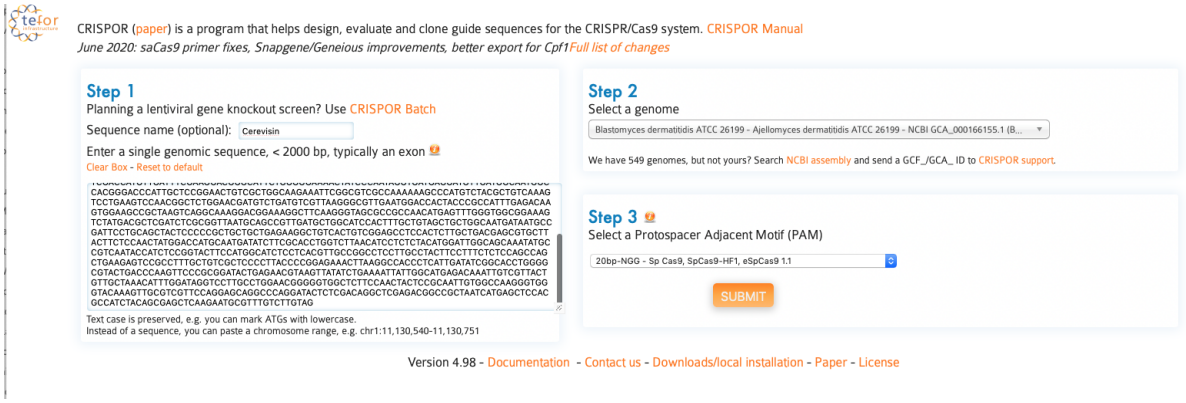
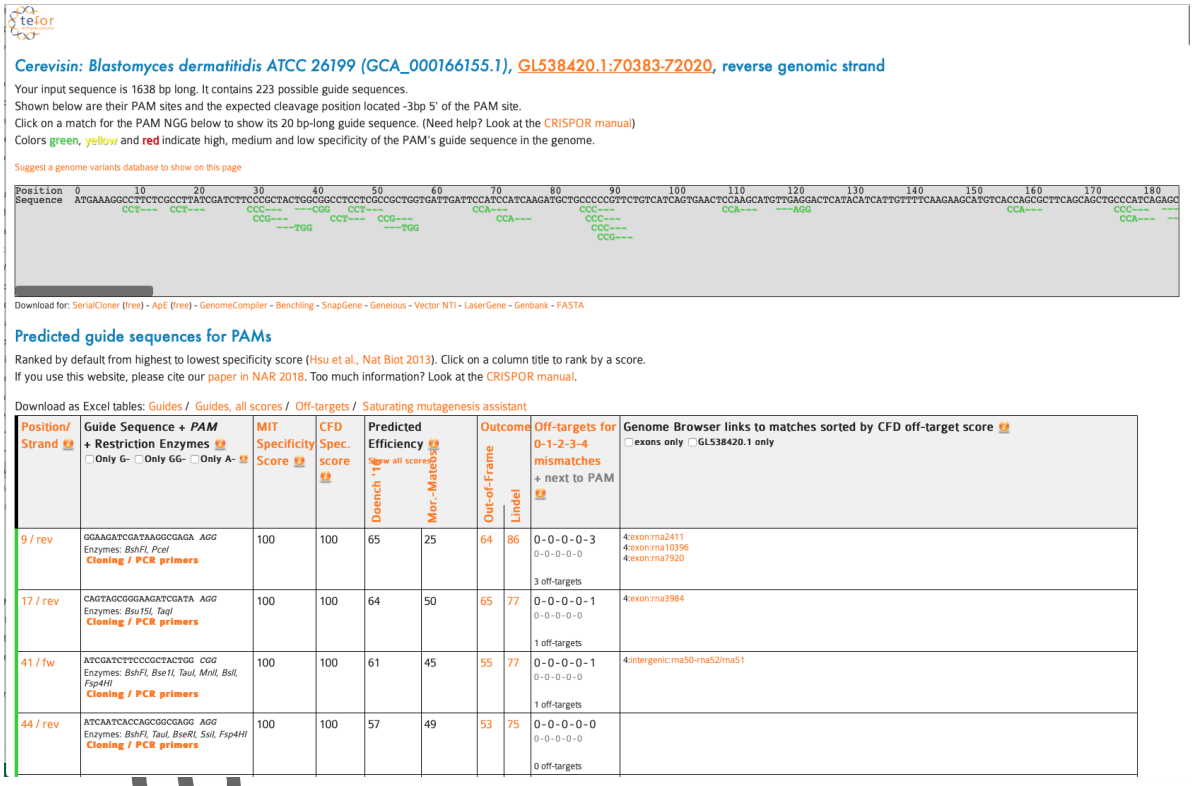


Figure 5: Example output of tefor CRISPOR sgRNA design tool.



tefor

Cerevisin: *Blastomyces dermatitidis* ATCC 26199 (GCA_000166155.1), GL538420.1:70383-72020, reverse genomic strand

Your input sequence is 1638 bp long. It contains 223 possible guide sequences.
Shown below are their PAM sites and the expected cleavage position located -3bp 5' of the PAM site.
Click on a match for the PAM NGG below to show its 20 bp-long guide sequence. (Need help? Look at the CRISPOR manual)
Colors green, yellow and red indicate high, medium and low specificity of the PAM's guide sequence in the genome.

Suggest a genome variants database to show on this page

Download for: SerialCloner (free) - ApE (free) - GenomeCompiler - Benchling - SnapGene - Geneious - Vector NTI - LaserGene - Genbank - FASTA

Predicted guide sequences for PAMs

Ranked by default from highest to lowest specificity score (Hsu et al., Nat Biot 2013). Click on a column title to rank by a score.
If you use this website, please cite our paper in NAR 2018. Too much information? Look at the CRISPOR manual.

Download as Excel tables: Guides / Guides, all scores / Off-targets / Saturating mutagenesis assistant

Position/ Strand	Guide Sequence + *PAM* + Restriction Enzymes	MIT Specificity Score	CFD Spec. score	Predicted Efficiency (Show all scores) Doench '16	Mor.-Mate65	Outcome Out-of-Frame	Lindel	Off-targets for 0-1-2-3-4 mismatches + next to PAM	Genome Browser links to matches sorted by CFD off-target score (exons only / GL538420.1 only)
9 / rev	GGAAGATCGATAAGGCGAGA *AGG* Enzymes: *BshFI, PceI* Cloning / PCR primers	100	100	65	25	64	86	0-0-0-0-3 0-0-0-0-0 3 off-targets	4:exon:rna2411 4:exon:rna10396 4:exon:rna7920
17 / rev	CAGTAGCGGGAAGATCGATA *AGG* Enzymes: *Bsu15I, TaqI* Cloning / PCR primers	100	100	64	50	65	77	0-0-0-0-1 0-0-0-0-0 1 off-targets	4:exon:rna3984
41 / fw	ATCGATCTTCCCGCTACTGG *CGG* Enzymes: *BshFI, Bse1I, TaqI, MnlI, BslI, Fsp4HI* Cloning / PCR primers	100	100	61	45	55	77	0-0-0-0-1 0-0-0-0-0 1 off-targets	4:intergenic:rna50-rna52/rna51
44 / rev	ATCAATCACCAGCGGCGAGG *AGG* Enzymes: *BshFI, TaqI, BseRI, SsiI, Fsp4HI* Cloning / PCR primers	100	100	57	49	53	75	0-0-0-0-0 0-0-0-0-0 0 off-targets	

Figure 6. **Timeline overview of culturing events for *Agrobacterium*-mediated transformation.** Red dots correspond to handling of *Agrobacterium* transformed with targeting vector. Blue squares indicate passaging of *Blastomyces* cultures in preparation for cocultivation. Green triangles represent cocultivation events. A convenient example schedule indicated by days of the week is shown above the timeline. AMM, *Agrobacterium*-minimal medium; IM+AS, induction medium with acetosyringone; EP, electroporation; Inoc., inoculation.

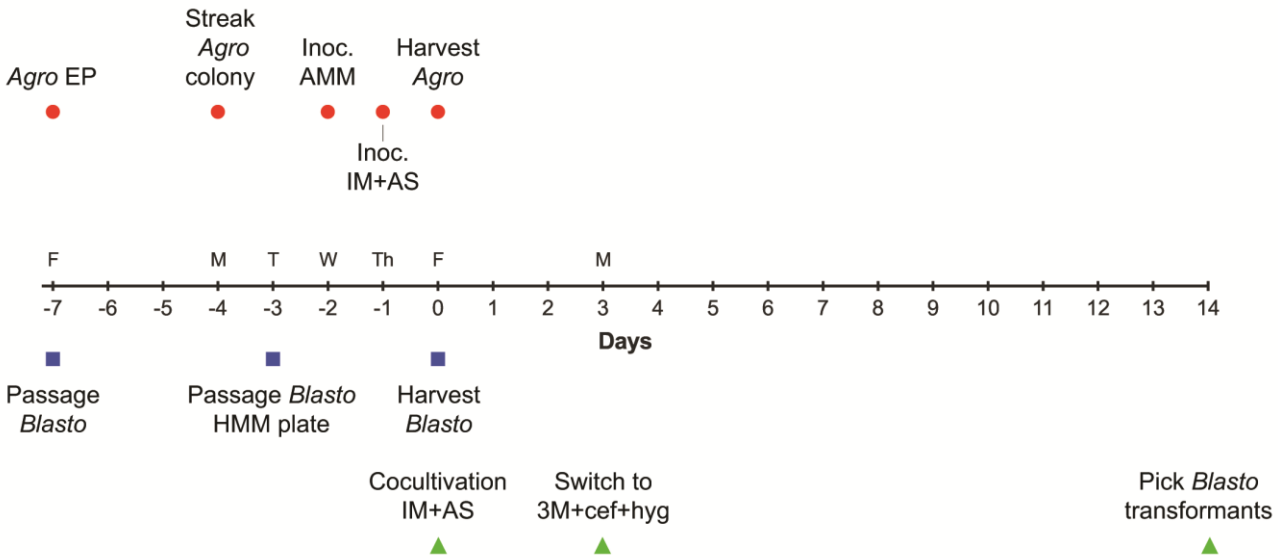


Figure 7. **Potential mechanisms for generation of insertions and deletions by DNA repair systems.** Double-stranded DNA breaks are commonly repaired by non-homologous end joining pathways. **(A)** Non-random small insertions are frequently biased by the base sequence immediately upstream of the Cas9 cleavage site. One potential mechanism to account for this bias involves asymmetric cleavage (black triangles) of the protospacer (green bar) upstream of the PAM (red), generating 1-nt overhangs that result in templated insertion of the duplicated base (green) after fill-in and ligation. **(B)** Small deletions are often associated with short regions of microhomology (blue) in and near the cleavage site. In this scenario, blunt-ends generated by Cas9 can be subjected to 5' end resection, allowing base-pairing of the short homologous sequences. Mismatched extruded sequences are removed and the resulting gap filled in and ligated, producing a short deletion (6 bp in this example). Note that the protospacer in this figure is on the bottom DNA strand. The region to be deleted is represented by the dashed line.

Table 1: PCR primers needed for sgRNA cloning

Primer	Sequence (5’ to 3’)	Notes
FragAF	ccgctgagggtttaatGCGTAAGCTCCCTAATTG	purple, Pgpd; gray, vector overlap for Gibson assembly. This primer is invariant and is used to generate multiple novel targeting vectors.
FragAR	GACGAGCTTACTCGTTTCGTCCTCACGGACTCATCAGGCTGGGCGGTGATGTCTGCTCAAG	New primer required for each target gene. Red, HH ribozyme includes 6 nt inverted repeat (IR; blue) complementary to 6 nt at the 5' start of protospacer—replace IR with gene-specific seq; purple, minus strand of Pgpd; underlined sequence represents overlap of fragments A &

B.

FragB F	GAAACGAGTAAGCTCGTCGCTGGGTTCCAATCTGCATGGTTTTAGAGCTAGAAA TAGC	New primer required for each target gene. Red underline, HH ribozyme, overlap with FragA R for Gibson assembly; target gene-specific 20 nt protospacer is represented by the blue (IR) and olive (remainder) sequences; dark green, invariant tracr RNA. NOTE: the IR is the same seq in the FragA R & FragB F primers, but the primers are for opposite strands, so in the assembled DNA, the 6-mers will be IR of each other.
FragB R	cggctgaggtcttaatGAGCCAAGAGCGGATTCC	Dark purple, TtrpC; gray, vector overlap

for Gibson assembly. This primer is invariant and is used to generate multiple novel targeting vectors.

Table 2: Example Cocultivation Setup

CC Group	No. of Plates	Blasto strain	No. of Blasto cells	Mix volume ratio	Agro transformant	Blasto+Agro Master mix volumes (each)	Notes
1	1–3	None	None	Agro only	pPTS608-Cas9-hyg	n/a	Shows efficacy of cefotaxime
2	1–3	26199*	10^7 yeast	Blasto only	none	n/a	Shows efficacy of hygromycin B
3	5	26199	10^7 yeast	1:1	pPTS608-Cas9-hyg	0.6 ml + 0.6 ml	Cas9-only controls
4	5	26199	10^7 yeast	1:1	pPTS608-Cas9-hyg-GOI sgRNA #1	0.6 ml + 0.6 ml	Gene-targeting (protospacer 1)
5	5	26199	10^7 yeast	1:1	pPTS608-Cas9-hyg-GOI sgRNA #2	0.6 ml + 0.6 ml	Gene-targeting (protospacer 2)

**Blastomyces dermatitidis* strain ATCC 26199
GOI, target gene of interest.