C. albicans Genetics Primer

Common Laboratory Strains

1. **General Information**
   a. The derivation of the common lab strains is nicely laid out on the Candida Genome Data Base (http://www.candidagenome.org/Strains.shtml#P37005). The most commonly used strains are derived from the clinical isolate SC5314. This strain is the basis for the most commonly used genome sequence, for which data on both alleles of most loci are now available.
   b. One of the most important considerations when choosing a genetic background is whether you plan to do mouse virulence studies. The presence of un-complemented auxotrophic markers can lead to virulence defects by themselves. The most famous and problematic example is the Uracil marker. The first genetic system for *C. albicans* was developed using a recyclable *URA3* marker using the CAI background (Ura-); it was a huge advance **(1)** but later studies showed that expression *of URA3* from ectopic positions in the genome did not fully complement **(2)**. Therefore, many virulence studies performed in the CAI background are confounded by the effect of ectopic *URA3* expression.
   c. If you are starting out in *C. albicans*, I would strongly advocate you adopt one of the more recently developed genetic backgrounds which have been extensively used in virulence studies.
2. **Specific Laboratory Strains**
   a. **CAI4**
      i. This strain was developed in the Fonzi lab **(1)** for use in "the URA-blaster" method of gene deletion based on disruption *URA3*-based cassettes followed by counter-selection with 5-fluoro-orotic acid (5-FOA).
      ii. Genotype: *ura3*::imm434/*ura3*::imm434 *iro1/iro1*::imm434
      iii. Name/genotype of strain with complemented auxotrophies: CAF2-1/*URA3/ura3*::imm434 *IRO1/iro1*::imm434.
      iv. Introduction of *URA3* at either the *RPS10* or *ENO1* loci leads to expression levels that support virulence comparable to CAF2-1 **(3)**.
   b. **BWP17**
      i. Derived from CAI4 by the Mitchell lab through the introduction of His and Arg auxotrophies **(4)**. This strain was used to develop PCR-based gene disruption in *C. albicans*. It has a Ura auxotrophy and is lacking a portion of chromosome 5.
      ii. Genotype: *ura3*::imm434/*ura3*::imm434 *iro1/iro1*::imm434 *his1*::hisG/*his1*::hisG *arg4/arg4*.
      iii. Name/genotype of strain with complemented auxotrophies: DAY185/ *ura3Δ::λimm434 ARG4:URA3:arg4::hisG his1::hisG::pHIS1*

iv. Complementation of these strains is most conveniently achieved by cloning the ORF into a *HIS1* containing plasmid by recombinational cloning in *S. cerevisiae* followed by integration at the *HIS1* loci **(5)**.

c. SN152

i. This background was derived from RM1000 and was specifically designed to avoid the use of URA markers. The *HIS1*, *LEU2* and *ARG4* genes were deleted using a PCR-based version of URA-blaster **(6)**. *URA3* was then re-integrated at its endogenous chromosomal position. The triple auxotrophic strain (SN152) has reduced virulence in mice but double auxotrophic strains (His-/Leu- (SN87) & His-/Arg- (SN95)) are indistinguishable from the parental RM1000.

ii. Genotype: *arg4Δ/arg4Δ leu2Δ/leu2Δ his1Δ/his1Δ URA3/ura3Δ::imm IRO1/iro1Δ::imm*

iii. The specific stain used for comparison/control depends upon the markers used to create the strain. Complementation using these strains has been typically performed by re-integration of a *LEU2* containing plasmid at the *RPS10* loci.

3. Clinical Isolates

a. SC5314 is the most commonly used laboratory strain that has not been genetically manipulated. SC5314 has been around a long time and has been passaged extensively. Therefore, not all SC5314 strains are equal. The use of the dominant drug selection marker *NAT1* which encodes for resistance toward the drug nourseothricin in the context of a recyclable cassette has allowed the direct generation of mutants in clinical isolates and it has been most widely applied to SC5314 **(7)**. This is a highly virulent strain and leads to rapid death in mice, particularly with inbred mice lines such as BALB/c.

b. ATCC 90028 is a strain available from ATCC that is less virulent than SC5314 and has been used by the Tom Patterson and his colleagues as part of the NIH contract lab for testing candidate anti-candidal drugs **(8)**.

4. Methods for the Construction of Gene Disruption Cassettes

a. General Principles

i. The most commonly employed approach **(4)** to generating deletion mutants is to create a deletion cassette that contains a auxotrophic or dominant drug marker flanked by sequence that is homologous to the 5' and 3' aspects of the region to be deleted (i.e., ORF). The specificity of the targeting increases with the length of the homologous sequences used as the flanking regions. In general, 100bp or more are required for this to be practical. This is much longer than those needed for *S. cereviae* where as few as 40bp are needed. The marker is either recycled or two different markers are used to generate a homozygous deletion mutant. For essential genes, one allele is deleted in this manner

and then the remaining allele can be put under the control of an inducible promoter **(9, 10, 11, 12).**

ii. The efficiency of transformation is much lower than *S. cerevisiae* so higher amounts of DNA are required (generally microgram quantities per transformation.

b. Cloning-based creation of cassette

i. The most common cloning-based approach to deletion cassette construction is through use of the SAT1-flipper cassette **(7)**. The SAT1 contains the *NAT1* marker as well as a maltose responsive promoter-driven CaFLP recombinase, although growth on YPD is generally sufficient to induce recombination. This allows induced removal of the *NAT1* maker for multiple rounds of disruption. The cassette has unique restriction sites that allow cloning 5' and 3' flanks to direct the cassette to the target.

c. PCR using long primers

i. This approach to generating cassettes is derived directly from *S. cerevisiae*. A set of primers are designed that contain approximately 100 bp of homology to the 5' end and 3' end of the sequence to be disrupted. This gene specific sequences flank sequences that will amplify the maker cassette from a plasmid template. The PCR products are then used to transform *C. albicans*. The Mitchell Lab pioneered this approach **(4)**. The most common markers are *URA3*, *HIS1*, *LEU2*, and *ARG4*. The Mitchell lab also constructed a *URA3* cassette with inverted repeats to facilitate counter-selection-based recycling as with the URA-blaster.

d. Fusion PCR

i. This approach to cassette construction used overlap extension or fusion PCR to increase the amount of gene specific sequence in order to improve targeting. Using this method, ~300 bp of sequence can be incorporated into the cassette. This method was used by the Johnson lab to create a set of deletion libraries in *C. albicans* **(6)**. It can be tricky to implement but leads to much improved efficiency with respect to specificity of transformation.

5. Transformation Methods

a. Lithium acetate

i. This is the most commonly used method and is based on the transformation protocol used for *S. cerevisiae*. The cells are exposed to a mixture of polyethylene glycol (PEG 3350)/LiOAc/single stranded carrier DNA and the transforming DNA usually overnight. Following heat shock, the cells are either directly plated on selective media (auxotrophic markers) or incubated in non-selective media as an outgrowth period (dominant drug resistance marker) for 3-24hr followed by plating **(13)**.

ii. This method of transformation is used to introduce the transient expression constructs for CRISPR/Cas9.

b. **Electroporation**
    i. This method is used by many laboratories. The cells are stabilized by the presence of 1M sorbitol. There is no carrier DNA. Pre-incubation in LiOAc or DTT has been reported to increased efficiency.
    ii. Electroporation is used to introduce Ca9 protein complexes for CRISPR.
c. **Spheroplast**
    i. Based on head-to-head comparisons, this is the most efficient method. The cell wall is degraded with a glucanase such as lyticase. This is somewhat tedious procedure but is very effective **(15).**

6. **Isolation and confirmation of mutants**
    a. Transformation efficiency varies with amount of DNA, strain background, and other various and sundry parameters. In general, you should see colonies within two or so days with most examples. If the mutant has a growth defect, then this will be longer. Be suspicious of mutants for which you only get one or two colonies, particularly when generating homozygous deletions---they may have additional mutations even if they confirm, especially if they appear to have significant growth defects.
    b. It is best to pick 8 or 10 colonies for confirmation. First, re-streak on selective plate to confirm the initial selection.
    c. PCR confirmation. Using primers that a gene specific sequence outside of the deletion cassette and a marker specific primer, confirm integration of the cassette to the correct place in genome.
    d. It is essential to confirm that a homozygous deletion mutant no longer contains a copy of the ORF. There are many examples of gene deletions that have triplication events leading to ectopic copies of the target ORF. If putative homozygous deletions repeatedly have ectopic copies of the ORF, then consider the possibility that the gene may be essential.
    e. You should bring multiple isolates forward at each stage and store a minimum of two independent clones of each mutant.
    f. Southern blots can also be used to confirm genotype and can be very useful if you run into strange results.
    g. RT-PCR can also be performed to insure that the ORF is missing.
    h. It is best to make glycerol stocks (YPD+20-30% glycerol) of your strains before confirmation. Storage of strains on plates for extended periods of time can lead to the accumulation of new mutations. Once mutant is confirmed and you are studying it do not leave it on the bench for extended periods of time. Make working stocks and re-streak every week or two. This will improve reproducibility.

7. **Complementation**
    a. The most convincing way to show that your phenotype is due to the genotype that you have engineered is to re-introduce a wild type copy of the gene and show that the phenotype is complemented.

b. Complementation requires re-integration of the gene into the chromosome in C. albicans because, unlike *S. cerevisiae*, no autonomously replicating episomal plasmid system has been discovered/developed.
c. A number of integrating plasmids have been developed for this purpose. Re-integration at an auxotrophic marker site such as *HIS1* or *LEU2* is very common. As noted above, re-integration of *URA3* based plasmids should be at either *ENO1* or at *RPS10* to give consistent expression **(3)**.
d. Re-integration at the endogenous position of the gene of interest can also be performed with non-*URA3*-based plasmids
e. The Berman lab has developed a set of plasmids that allow integration of the gene into an "empty" portion of the genome **(16)**.
f. One convenient set of plasmids developed by both the Mitchell **(5)** and Berman **(16)** labs allow one to clone the ORF into a plasmid using *S. cerevisiae* as an intermediate organism through recombinational cloning. This is restriction enzyme free cloning and is very efficient.
g. It is important to realize that complementation does not always restore the phenotype to WT. This can be due to positional effects on expression or may be due to haploinsufficiency—a phenotype associated with the heterozygote.
h. An alternative to complementation is to isolate and evaluate multiple independent strains with the same genotype. The rationale for this approach is that identical random background mutations are unlikely to occur in two or more independent transformation events.

8. **Gene Deletion Sets**
    a. **General**
        i. A variety of libraries of deletion mutants have been constructed by members of the *C. albicans* community as well as Merck Labs. Many of these are available through the Fungal Genetic Stock Center **(17)**. It is important to confirm the genotype of any strains that you study since contamination of wells can lead to mixing of strains. If you are going to extensively study a gene based on initial library screening results, it is probably best to re-make the strain independently. These libraries are not flawed but passaging and contamination can affect reproducibility; it is best to be safe rather than sorry and embarrassed.
        ii. **Deletion Libraries**
            1. **Homann Transcription Factor Deletion Library:** 154 homozygous deletion mutants of transcriptional regulators in the SN background **(18)**; these are Leu+/His+/Arg-. The mutants are complete deletions of the ORFs. They have been extensively characterized by the Johnson Lab and have been screened by many others in the community.
            2. **Noble deletion set:** This is a set of strains generated in the same way as the Homann Library but involve a wide range of ORFs with different functions **(19)**.

3. **Mitchell Kinase and Transcription Factor Libraries:** These are libraries that were generated using a transposon-based homozygous deletion strategy in the BWP background **(5, 20)**. These are not homozygous deletion mutants and thus phenotypes may not match a deletion exactly.
4. **Merck-barcoded heterozygous deletion library:** This is a set of 5127 barcoded deletion heterozygous deletion mutants that were developed for chemical-induced haploinsufficiency assays. These are now housed with Leah Cowen's lab in Toronto **(21).**

9. **CRISPR/Cas9**
   a. Since the initial application of CRISPR/Cas9 to *C. albicans* using integration of the components was reported **(22)**, a number of approaches and tools for CRISPR/Cas9 gene editing have been developed for use in *C. albicans* as well as other Candida spp. The two most widely used are a transient expression-based system developed by Aaron Mitchell's lab (**23**) and, more recently, direct introduction of Cas9 protein-based complexes into non-albicans Candida spp. (**24**).
   b. The major advance that CRISPR/Cas9 approaches offer for *C. albicans* genetics is that both alleles of a gene can be disrupted in a single transformation. In addition, multiple gene disruptions can be executed in a single transformation, although the efficiency drops as the number of independent editing events is combined.
   c. We are using the transient system of the Mitchell lab in the course this year so additional details are provided in the lab syllabus.

References

1. **Fonzi WA, Irwin MY.** 1993. Isogenic strain construction and gene mapping in Candida albicans. **Genetics** 134:717-28.
2. **Lay J, Henry LK, Clifford J, Koltin Y, Bulawa CE, Becker JM**. 1998. Altered expression of selectable marker *URA3* in gene-disrupted *Candida albicans* strains complicates interpretation of virulence studies. **Infect Immun** 66:5301-6.
3. **Brand A, MacCallum DM, Brown AJ, Gow NA, Odds FC.** 2004. Ectopic expression of *URA3* can influence the virulence phenotypes and proteome of *Candida albicans* but can be overcome by targeted reintegration of *URA3* at the *RPS10* locus. **Eukaryot Cell.** 3:900-9.
4. **Wilson RB, Davis D, Mitchell AP.** 1999. Rapid hypothesis testing with *Candida albicans* through gene disruption with short homology regions. **J Bacteriol.** 181(6):1868-74.

5. **Blankenship JR, Fanning S, Hamaker JJ, Mitchell AP.** 2010. An extensive circuitry for cell wall regulation in *Candida albicans*. **PLoS Pathog**. 6:e1000752.
6. **Noble SM, Johnson AD.** 2005. Strains and strategies for large-scale gene deletion studies of the diploid human fungal pathogen *Candida albicans*. **Eukaryot Cell** 4:298-309.
7. **Sasse C, Morschhäuser J.** 2012. Gene deletion in *Candida albicans* wild-type strains using the SAT1-flipping strategy. **Methods Mol Biol.** 845:3-17.
8. **http://www.niaid.nih.gov/LabsAndResources/resources/dmid/animalModels/SOPs/Documents/SOP-murine-model-candida-albicans.pdf.**
9. **Michel S, Ushinsky S, Klebl B, Leberer E, Thomas D, Whiteway M, Morschhäuser J.** 2002. Generation of conditional lethal *Candida albicans* mutants by inducible deletion of essential genes. **Mol Microbiol**. 46:269-80.
10. **Care RS, Trevethick J, Binley KM, Sudbery PE.** 1999. The *MET3* promoter: a new tool for *Candida albicans* molecular genetics. **Mol Microbiol.** 34:792-8.
11. **Weyler M, Morschhäuser J.** 2012. Tetracycline-inducible gene expression in Candida albicans. **Methods Mol Biol.** 845:201-10.
12. **Wilson RB, Davis D, Enloe BM, Mitchell AP.** 2000. A recyclable *Candida albicans URA3* cassette for PCR product-directed gene disruptions.Yeast. **16:**65-70.
13. **Walther A, Wendland J.** 2003. An improved transformation protocol for the human fungal pathogen *Candida albicans*. **Curr Genet.** 42:339-43.
14. **De Backer MD, Maes D, Vandoninck S, Logghe M, Contreras R, Luyten WH.** 1999. Transformation of *Candida albicans* by electroporation. **Yeast** 15:1609-18.
15. **Herreros E, García-Sáez MI, Nombela C, Sánchez M.** 1992. A reorganized Candida albicans DNA sequence promoting homologous non-integrative genetic transformation. **Mol Microbiol.** 6:3567-74.
16. **Gerami-Nejad M, Zacchi LF, McClellan M, Matter K, Berman J. 2013.** Shuttle vectors for facile gap repair cloning and integration into a neutral locus in *Candida albicans*. **Microbiology** 159:565-79.
17. **http://www.fgsc.net/**
18. **Homann OR, Dea J, Noble SM, Johnson AD.** 2009. A phenotypic profile of the Candida albicans regulatory network. **PLoS Genet.** 5:e1000783.
19. **Noble SM, French S, Kohn LA, Chen V, Johnson AD**. 2010. Systematic screens of a *Candida albicans* homozygous deletion library decouple morphogenetic switching and pathogenicity. **Nat Genet.** 42:590-8.
20. **Finkel JS, Xu W, Huang D, Hill EM, Desai JV, Woolford CA, Nett JE, Taff H, Norice CT, Andes DR, Lanni F, Mitchell AP.** 2012. Portrait of Candida albicans adherence regulators. **PLoS Pathog.** 8:e1002525.
21. **Xu D, Jiang B, Ketela T, Lemieux S, Veillette K, Martel N, Davison J, Sillaots S, Trosok S, Bachewich C, Bussey H, Youngman P, Roemer T.** 2007. Genome-wide fitness test and mechanism-of-action studies of inhibitory compounds in *Candida albicans.* **PLoS Pathog**. 3:e92.
22. **Vyas VK, Barrasa MI, Fink GR**. 2015. A *Candida albicans* CRISPR system permits genetic engineering of essential genes and gene families. **Sci Adv** 1:e1500248.

23. **Min K, Ichikawa Y, Woolford CA, Mitchell AP.** 2016. Candida albicans Gene Deletion with a Transient CRISPR-Cas9 System**. mSphere.** 1. pii: e00130-16.
24. **Grahl N, Demers EG, Crocker AW, Hogan DA.** 2017**.** Use of RNA-Protein Complexes for Genome Editing in Non-*albicans Candida* Species. mSphere 2(3). pii: e00218-17.