CHAPTER THIRTY-ONE

Genetics and Molecular Biology in *Candida albicans*

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Methods in Enzymology, Volume 470
ISSN 0076-6879, DOI: 10.1016/S0076-6879(10)70031-8

Abstract

Candida albicans is an opportunistic fungal pathogen of humans. Although a normal part of our gastrointestinal flora, *C. albicans* has the ability to colonize nearly every human tissue and organ, causing serious, invasive infections. In this chapter we describe current methodologies used in molecular genetic studies of this organism. These techniques include rapid sequential gene disruption, DNA transformation, RNA isolation, epitope tagging, and chromatin immunoprecipitation. The ease of these techniques, combined with the high-quality *C. albicans* genome sequences now available, have greatly facilitated research into this important pathogen.

Candida albicans is a normal resident of the human gastrointestinal tract; it is also the most common fungal pathogen of humans, causing both mucosal and systemic infections, particularly in immune compromised patients. *C. albicans* and *Saccharomyces cerevisiae* last shared a common ancestor more than 900 million years ago; in terms of conserved coding sequences, the two species are approximately as divergent as fish and humans. Although *C. albicans* and *S. cerevisiae* share certain core features, they also exhibit many significant differences. This is not surprising as *C. albicans* has the ability to survive in nearly every niche of a mammalian host, a property not shared by *S. cerevisiae*. Research into *C. albicans* is important in its own right, particularly with regards to its ability to cause disease in humans; in addition, comparison with *S. cerevisiae* can reveal important insights into evolutionary processes.

Many of the methodologies developed for use in *S. cerevisiae* have been adapted for *C. albicans*, and we describe some of the most common. Although alternative procedures are described in the literature, we have found those described below to be the most convenient. Because the *C. albicans* parasexual cycle is cumbersome to use in the laboratory, genetics in this organism has been based almost entirely on directed mutations. Because the organism is diploid, creating a deletion mutant requires two rounds of gene disruption. We describe a rapid method for creating sequential disruptions, one which can be scaled up to create large collections of *C. albicans* deletion mutants. We also describe a series of additional techniques including DNA transformation, mRNA isolation, epitope tagging, and chromatin immunoprecipitation (ChIP). The ease of these techniques, combined with the high-quality *C. albicans* genome sequences now available, has greatly increased the quality and pace of research into this important pathogen.

1. Homozygous Gene Disruption in *C. albicans*

Creating gene knockout mutants in *C. albicans* typically involves two rounds of transformation (to disrupt both alleles of a given gene) with a linear fragment of DNA bearing a selectable marker as well as sequences identical (or nearly identical) to those sequences flanking the target gene

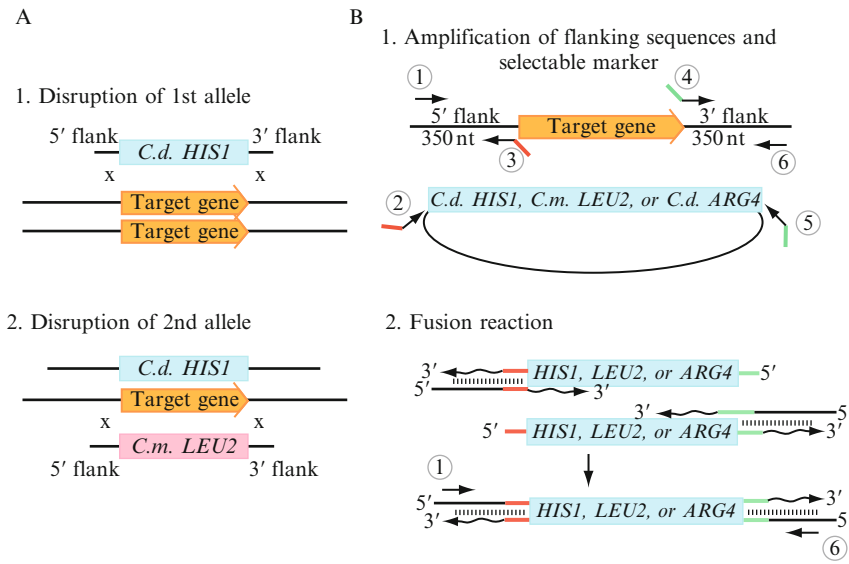


Figure 31.1 (A) Homozygous gene disruption by two rounds of transformation and homologous recombination. (B) Fusion PCR method.

(Fig. 31.1A). Approximately 60 nucleotides of flanking sequence on each side of the selectable marker approaches the minimum necessary for successful targeting, and the efficiency appears to improve with increasing lengths. The disruption cassette can be created by PCR or by traditional cloning, and the available selectable markers include multiple auxotrophic markers (such as *HIS1*, *LEU2*, *ARG4*, and *URA3*) and an antibiotic resistance gene (*SAT1*); note that the *URA3* marker should be used with care, because important *C. albicans* phenotypes such as morphogenesis and virulence are strongly dependent on the levels of *URA3* expression. Transformants are selected on appropriate media and then screened for integration of the disruption cassette at the correct genomic locus. Following disruption of the second allele, verification that the target ORF is truly deleted (achieved by PCR or Southern blotting) is crucial, as extra copies of chromosomes can arise during the tranformation procedures.

Following is a streamlined protocol based on fusion PCR that results in a high efficiency of gene disruption (Noble and Johnson, 2005; Fig. 31.1B). Maximal efficiency is achieved by the use of auxotrophic markers from non-*albicans Candida* species or bacterial antibiotic resistance genes as markers, these strategies reduce integration events at "off-target" locations in the *C. albicans* genome. The first round of PCR consists of three reactions: two to amplify DNA upstream and downstream of the target gene, and a third to amplify the selectable marker. Importantly, certain primers (indicated in Fig. 31.1 and described in detail in the protocol) contain complementary tails. In the second

round of PCR, the three products of the first round of PCR serve as an aggregate template, resulting in a single product. Note that specific reagents and kits are recommended, but alternates can be substituted. We have found that Ex Taq (Takara) and Klentaq LA (DNA Polymerase Technology) yield better results than other commercial enzymes for fusion PCR.

1.1. Homozygous gene disruption by fusion PCR

The following auxotrophic markers are available as cloned genes from non-*albicans Candida* species (Noble and Johnson 2005):

C. dubliniensis HIS1 = pSN52
C. maltosa LEU2 = pSN40
C. dubliniensis ARG4 = pSN69

1. Design the following PCR primers:

   Gene disruption primers (Fig. 31.1B):

   1—primer to gene of interest (beginning of 5′ flank top, ~350 bp upstream of ORF)
   2★—CCGCTGCTAGGCGCGCCGTG-selectable marker (5′ marker top)
   3—CACGGCGCGCCTAGCAGCGG-gene of interest (end of 5′ flank bottom)
   4—GTCAGCGGCCGCATCCCTGC-gene of interest (beginning of 3′flank top)
   5★—GCAGGGATGCGGCCGCTGAC-selectable marker (3′ marker bottom)
   6—primer to gene of interest (end of 3′ flank bottom, ~350 bp downstream of ORF)

★ If using auxotrophic markers in the pSN series (Noble and Johnson), sequences of primer 2 and primer 5 are:

Primer 2	ccgctgctaggcgcgccgtgACCAGTGTGATGGATATCTGC
Primer 5	gcagggatgcggccgctgacAGCTCGGATCCACTAGTAACG

   Knockout verification primers (Fig. 31.2):

   Target Check Left—just upstream of primer 1
   Target Check Right—just downstream of primer 6
   ORF Left—internal to the deleted ORF
   ORF Right—internal to the deleted ORF
   Marker Check Left★—toward end of selectable marker that is near primer 2
   Marker Check Right★—toward end of selectable maker that is near primer 5

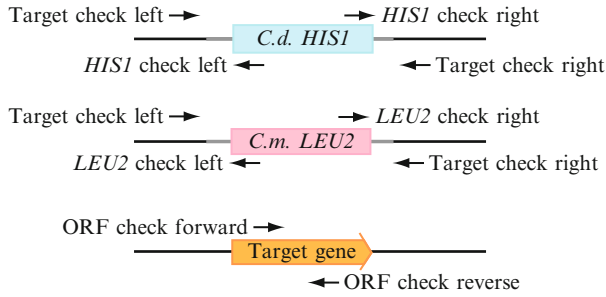


Figure 31.2 **Primers for verification PCR.**

*If using auxotrophic markers in the pSN series, one can use the following Marker Check primers:

C. dubliniensis HIS1 Check Left	ATTAGATACGTTGGTGGTTC
C. dubliniensis HIS1 Check Right	AACACAACTGCACAATCTGG
C. maltosa LEU2 Check Left	AGAATTCCCAACTTTGTCTG
C. maltosa LEU2 Check Right	AAACTTTGAACCCGGCTGCG
C. dubliniensis Check Left	TTCAACCTTTCAAACGATGC
C. dubliniensis Check Right	TCGATACATTTGCGGTACAG

2. Set up reactions for *PCR Round I* on ice, and run the PCR:

   Reaction 1 = primers 1 + 3 using genomic DNA as template
   Reaction 2 = primers 4 + 6 using genomic DNA as template
   Reaction 3 = primers 2 + 5 using auxotrophic marker as template, for example, pSN52 = *C. dubliniensis* HIS1

   PCR reaction (50 µl):

   5 µl 10× Ex Taq buffer
   4 µl 2.5 m*M* dNTPs
   36 µl H_2O
   < 1 µl GENOMIC DNA (reactions 1 and 2) or < 1 µl plasmid DNA (reaction 3)
   2 µl 1st primer (5 µ*M*)
   2 µl 2nd primer (5 µ*M*)
   0.5 µl Ex Taq polymerase

   PCR conditions:

   94 °C 5 min
   35 cycles 94 °C 30 s, 45 °C 45 s, 72 °C 1 min (flanks) or 4 min (marker)
   72 °C 10 min
   10 °C forever

3. Run out 5 µl PCR products on a 1% agarose gel to confirm successful synthesis.

4. Optional—Gel purify the product of Reaction 3 (marker fragment). Run the PCR reaction on a 1% agarose gel and cut out the correct sized band under long wave ultraviolet light. Recover DNA with a Qiagen QIAquick gel extraction kit (eluting in 50 μl H_2O or Buffer EB). By isolating the correct PCR product and eliminating contaminants, this step increases the efficiency of the fusion reaction for certain targets and allows for stable storage of the marker fragment (at –20 °C).
5. Optional—Purify the products of Reactions 1 and 2 with a Qiagen QIAquick PCR purification kit. Use as directed and elute in 50 μl H_2O or Buffer EB.
6. Set up reaction for *PCR Round II* on ice, and run PCR.

   PCR reaction (100 µl):

   10 μl 10× Ex Taq buffer
   8 μl 2.5 m*M* dNTPs
   0.75 μl Ex Taq polymerase
   67 μl H_2O
   1.5 μl reaction 1 PCR product (5′ flank)
   1.5 μl reaction 2 PCR product (3′ flank)
   2 μl reaction 3 PCR product (Marker)
   4 μl primer 1 (5 μM)
   4 μl primer 6 (5 μM)

   Fusion PCR conditions:

   94 °C 5 min (Hot start: i.e., wait until the PCR block heats to ~80 °C before introducing PCR reactions)
   35 cycles 94 °C 30 s, 50 °C 45 s, 72 °C 4.5 min
   72 °C 10 min
   10 °C forever

7. Run out 5 μl PCR product on a 1% agarose gel to confirm success of fusion reaction.

   The fusion product should be ~3–4 kb, depending on the auxotrophic marker chosen and the length of flanking sequences. There is typically a mix of full sized product, with a variable amount of a minority shorter product.

   Note: If the fusion reaction is unsuccessful, a variation is to include Betaine in the reaction; that is, add 20 μl of 5 *M* betaine (final 1.3 *M*) and just 47 μl H_2O in the fusion reaction mix. If betaine is used, decrease the PCR denaturation temperature to 92.5 °C.
8. Optional—Purify the fusion PCR product with a Qiagen QIAquick PCR purification kit.

   Use kit as directed and elute in 30 μl of H_2O or Buffer EB.

9. Transform 10 μl of disruption fragment into fresh competent SN152 (or any strain that is auxotrophic for the selectable marker). Plate cells on the appropriate dropout plate, for example, -His. Incubate plates at 30 °C for 2 days, or until individual colonies are visible.

   Note: If selecting for Nourseothricin resistance, allow cells to recover by growing in YPD liquid media without selection for at least 5 hours at 30 °C prior to plating on selective media.
10. Patch ~10 transformants onto fresh medium, and perform colony PCR to verify the correct 5′ and 3′ junctions of the disrupted allele (Fig. 31.2). In separate PCR reactions, use the primer pairs Target Left + Marker Left and Target Right + Marker Right. Correct integrants should have PCR products of the expected size (~0.5 kb) with each primer set.
11. Pick at least two confirmed heterozygous knockout candidates and streak for single colonies on fresh medium.

    Note: Because unlinked mutations can be acquired during strain construction and because two rounds of transformation are required to create a homozygous deletion, it is wise to obtain at least 2 independent isolates of any *C. albicans* knockout.
12. Repeat the fusion PCR step, using the same flank products (PCR reactions 1 and 2) but a different selectable marker (e.g., pSN40 = *C. maltosa* LEU2).
13. Transform 10 μl of the new disruption fragment into two independent heterozygous knockout strains and plate on doubly selective medium (e.g., -His, -Leu).
14. Patch ~10 transformants of each strain onto fresh medium, and perform colony PCR as above to confirm the appropriate 5′ and 3′ junctions of the second disrupted allele.
15. For candidates with expected disruptions of both target alleles, perform a final PCR verification that there are no remaining copies of the target ORF. This step is necessary because aneuploidies or translocations commonly result in an extra copy of the target gene. Remember to test as a positive control a strain that retains a copy of the target gene (e.g., wild type or the heterozygous knockout). One should see a PCR product of the expected size in the positive control and no PCR product in the desired homozygous deletion strain.

2. *C. albicans* DNA Transformation

The following is a basic protocol for DNA transformation with *C. albicans*. Because stable extrachromosomal plasmids have yet to be fully developed for use in *C. albicans*, this protocol is typically used for transformation and stable integration of linear DNA fragments into the *C. albicans*

genome. For efficient homologous recombination to occur, a minimum of 60 bp of sequence identical (or nearly identical) to the genomic target locus is required on either end of the DNA fragment that is to be transformed.

1. Inoculate a 5-ml culture in YEPD and grow overnight at 30 °C.
2. Dilute 300 μl of the overnight culture into 10 ml of fresh YEPD and grow at 30 °C for 4–6 h (or until OD is around 0.5–1.0).
3. Centrifuge for 2 min at ~1000×*g* and discard supernatant.
4. Resuspend in 900 μl LiOAc/TE and transfer to a microcentrifuge tube.
5. Pellet for 1 min at ~1000×*g* and discard supernatant.
6. Wash two more times with 900 μl LiOAc/TE then resuspend in ~400 μl final volume with LiOAc/TE.
7. In a separate microfuge tube mix (in order) the following:
   a. 10 μl 10 mg/ml denatured Herring Sperm (or Salmon Sperm) DNA
      i. Prepare by boiling ~2 min then snap cooling in ice water
   b. ≥1 μg of DNA fragment to be transformed (or ~20–50 μl of PCR product)
      ii. Highest transformation efficiencies are achieved if the DNA is NOT purified following enzymatic reactions (i.e., PCR products or plasmid digests)
   c. 200 μl washed cells in LiOAc/TE
   d. 1 ml PEG mix
8. Incubate overnight at room temperature.
9. Heat shock 1 h at 42 °C (or 44 °C for 15 min).
10. Pellet for 1 min at ~1000×*g* and discard supernatant.
11. Wash one time with 1 ml sterile water.
12. Resuspend in ~150 μl final volume with sterile water.
    a. For selection of Nourseothricin resistance, transfer cells to 5 ml YEPD and recover by incubation at 30 °C for at least 5 h prior to plating on selective media.
13. Plate on selective media and incubate at 30 °C for 2–3 days.

2.1. Transformation buffers

LiOAc mix:

10 ml 1 *M* LiOAc
200 μl 0.5 *M* EDTA
1 ml 1 *M* Tris–HCl, pH 7.5
H_2O to 100 ml
Filter sterilize

PEG mix:

80 ml 50% PEG-3350
10 ml 1 *M* LiOAc

200 μl 0.5 *M* EDTA
1 ml 1 *M* Tris–HCl, pH 7.5
H_2O to 100 ml
Filter sterilize

3. *C. albicans* Total RNA Purification

Purifying total cellular RNA from liquid cultures of *C. albicans* is comparable in most regards to purifications from *S cerevisiae*. As with *S. cerevisiae*, lysing the *C. albicans* cell wall requires a more vigorous procedure than does lysis of animal cells. The procedure outlined below includes organic extractions in Phase Lock tubes (Eppendorf) for removal of proteins and other cellular material. Because *C. albicans* cellular debris tends to disrupt the Phase Lock gel matrix, the first organic extraction is performed in conventional rather than Phase Lock tubes; if desired, subsequent extraction steps may also be performed in conventional tubes. Purified RNA is suitable for most applications, including microarray analysis, quantitative RT-PCR and Northern hybridization.

1. Grow a 10-ml liquid culture of *C. albicans* cells to an appropriate concentration (e.g., OD_{600} of 1.0–1.5). For other volumes and cell densities, all steps may be scaled proportionally.
2. Collect cells by centrifugation (2000×*g*, 5 min, 4 °C) in a 15-ml polypropylene conical tube. Remove as much liquid as possible, and freeze by immersing tube in liquid nitrogen. Store frozen cell pellet at −80 °C.
3. Transfer frozen tube containing cell pellet to ice, working quickly to avoid thawing prior to the addition of phenol.
4. To frozen pellet, first add 2 ml phenol, then 2 ml extraction buffer (50 m*M* sodium acetate [from pH 5.3 stock], 10 m*M* EDTA and 1% SDS). The use of acidic (pH ~4.5) rather than neutral phenol will reduce, but not eliminate, DNA contamination. *While working with phenol and chloroform, use appropriate protective equipment (goggles, gloves, lab coat, fume hood) and dispose of hazardous waste appropriately.*
5. Ensure that each tube is well-capped, then mix by vortexing. Transfer tube to 65 °C water bath. Incubate for 10 min, removing to vortex vigorously every minute or so.
6. Transfer tube to ice for 5 min. Keep samples on ice for all subsequent steps, except where noted.
7. Add 2 ml chloroform to tube, cap securely, then mix well by vortexing.
8. Spin tube in tabletop centrifuge (2000×*g*, 5 min, 4 °C) to separate phases, along with an empty 15 ml Heavy Phase Lock Gel tube (Eppendorf) for use in the next step.

9. Carefully remove aqueous (top) phase by pipetting, avoiding as much material at the interface as possible. Transfer to the prespun Phase Lock tube along with 2 ml phenol:chloroform. (Use an equal volume mixture of phenol and chloroform; this can be either neutral or acidic, with or without isoamyl alcohol.) Cap tube and shake by hand, but do not vortex, as this may disrupt the gel matrix.
10. Spin tube in tabletop centrifuge (1500×*g*, 5 min, 4 °C). Organic phase should partition below the gel matrix.
11. Add 2 ml chloroform, then shake and spin as before. (This step removes residual phenol from the sample.)
12. From this point on, ensure that all reagents and containers are free of RNases. Pour aqueous phase into a fresh conical tube. Add 200 μl 3 *M* sodium acetate (pH 5.3) and 2 ml isopropyl alcohol. Shake or vortex briefly, then incubate at room temperature for 10 min.
13. Pellet RNA in tabletop centrifuge at maximum speed (20 min, 4 °C). A substantial white pellet of RNA should be visible. Pour off supernatant and let drain briefly with tube inverted on a Kimwipe.
14. Use 800 μl 70% ethanol to transfer pellet by pipet to a 1.5-ml microfuge tube, breaking up pellet if necessary. Spin in 4 °C microcentrifuge at maximum speed for 5 min.
15. Carefully remove as much liquid as possible from pellet with pipet tip, then air dry for a few minutes. Do not allow RNA pellet to become too dry, as this will make resuspension difficult.
16. Resuspend RNA in ~200 μl RNase-free water. Pipetting up and down will help to resuspend the RNA; if necessary, the tube can also be incubated at 50 °C for 10 min.
17. To determine the concentration of RNA in solution, measure its absorbance in an ultraviolet spectrophotometer. If using a NanoDrop (Thermo Scientific), 2 μl of solution can be measured directly. Otherwise, dilute 1:100 to measure. The concentration of the measured solution (in ng/μl) is given by the absorbance at 260 nm multiplied by 40. Expected yield is roughly 400 μg.
18. RNA can be stored at −80 °C, then thawed slowly on ice for use. For downstream applications that may be compromised by contaminating DNA (such as quantitative RT-PCR), RNA should first be treated with an RNase-free DNase (e.g., RQ1 DNase from Promega).

4. C-Terminal Epitope Tagging in *C. albicans*

This protocol relies on homologous recombination to integrate the coding sequence for a C-terminal epitope tag in place of the stop codon for any gene at its endogenous locus. The pADH34 vector contains the coding

sequence for a 13× myc repeat, while pADH52 encodes a 6-His/FLAG tandem affinity purification (TAP) tag. As both of these constructs use the same linker sequence, either tag can be amplified with a single pair of PCR primers. Briefly, long oligonucleotides (typically 90–120 bp total) are used to amplify a 4.8-kb DNA fragment which, when integrated into the genome, will replace the stop codon of the target gene with the epitope tag coding sequence, followed by the SAT1/flipper cassette. Upon confirmation of integration at the desired locus in nourseothricin resistant colonies, the SAT1/flipper cassette is excised, leaving only the epitope tag coding sequence and a minimally disruptive FLP recombinase target sequence behind. The SAT1/flipper cassette and marker excision procedure was developed by Reuss *et al.* (2004).

4.1. Primer design

Synthesize a "forward knock-in primer" encompassing the sense strand sequence of the target gene up to, but not including, the stop codon. In place of the stop codon, add the sequence "CGGATCCCCGGGTTAATTAACGG" to the 3′ end of the forward knock-in primer. To generate the "reverse knock-in primer," take the reverse complement of the sequence immediately downstream of the stop codon and add the sequence "GGCGGCCGCTCTAGAACTAGTGGATC" to the 3′ end.

4.2. PCR conditions

Perform 30–35 cycles of amplification with pADH34 or pADH52 and the knock-in primers using Ex Taq (Takara) or a similar increased fidelity/high-activity thermostable polymerase. Use a three-step program for the first five cycles, with annealing at 58 °C and 5 min extensions at 72 °C. For the remaining cycles perform a 2-step program, eliminating the annealing step. To minimize the chances of acquiring PCR generated mutations in the knock-in cassette, perform three independent PCR reactions for each target gene and pool the reactions following amplification.

4.3. Transformation

Directly transform ~20–50 μl of the knock-in cassette PCR product (without purification) into the target strain using standard *C. albicans* transformation methods as described above. Following transformation, but prior to selection, wash the cells twice with YEPD then split to two independent 5 ml cultures (to insure isolation of independent clones) and recover for at least 5 h at 30 °C. Pellet and plate the entire culture onto YEPD + 400 μg/ml nourseothricin. *Note*: addition of adenine and/or uridine to the growth medium, even with prototrophic strains, can increase the efficiency of several steps in this protocol.

4.4. Integration confirmation

Screen nourseothricin resistant colonies by colony PCR with the following primers:

Upstream flank check: Use a primer that hybridizes ~500 bp upstream of the target gene stop codon (extending toward the stop codon) and AHO300. (CCGTTAATTAACCCGGGGATC). AHO300 anneals to the linker sequence, which is common to both pADH34 and pADH52, and extends into the tagged ORF.

Downstream flank check: Use a primer that hybridizes ~500 bp downstream of the target gene stop codon (extending toward the stop codon) and AHO301 (GGAACTTCAGATCCACTAGTTCTAGAGC), which anneals to both pADH34 and pADH52.

4.5. SAT1 marker excision

To induce excision of the SAT1/flipper cassette, culture nourseothricin resistant strains in YEP-maltose (2%) for at least 5 h (or overnight) at 30 °C and plate ~100 cells/plate on YEPD + 25 μg/ml nourseothricin. (Note that some mutant strains are hypersensitive to nourseothricin, and lower concentrations (≤5 μg/ml) may be necessary.) Following 1–2 days of growth at 30 °C, small, medium, and large colonies should be observed. Patch small and medium sized colonies on to YEPD + 400 μg/ml nourseothricin and onto YEPD without selection to screen for nourseothricin sensitive colonies. To confirm excision of the SAT1/FLP cassette, perform colony PCR with either AHO302 (TCACTAGTGAATTCGCGCTCGAG, for myc tagging with pADH34) or AHO405 (TAAATAATGAATTCGCGCTCGAG, for TAP tagging with pADH52) and the downstream flank check primer described above.

4.6. Tag sequence confirmation

To confirm that the target ORF and the epitope tag are free of mutations, perform colony PCR using a high fidelity polymerase and the following primers: AHO283 (GGCGGCCGCTCTAGAACTAGTGGATC, common to both pADH34 and pADH52) and the upstream flank check primer designed above. AHO283 anneals to the 3′ end of the residual SAT1/FLP cassette sequence (including the FRT) and extends toward the tagged gene. Following colony PCR amplification from the tagged strain, purify the PCR product and sequence with AHO283 as the sequencing primer.

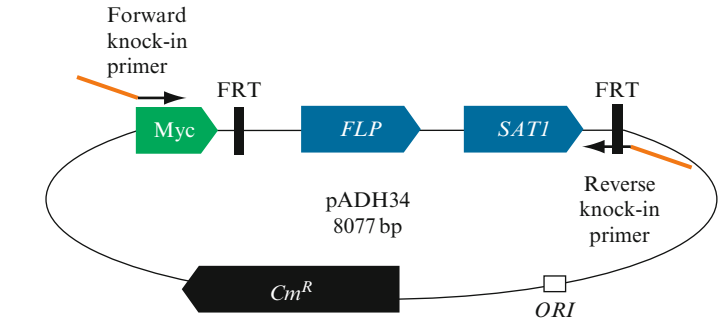
Note: The pADH34 myc tagging construct inserts 629 bp in place of the original stop codon, while the pADH52 TAP tagging construct inserts 152 bp. To determine the expected size of the PCR product for

sequencing, add this number to the distance between the upstream flank check primer and the original stop codon of the target gene.

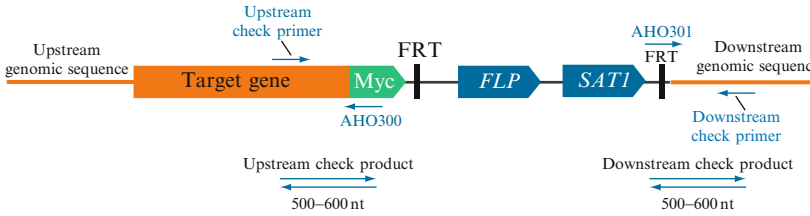
4.7. Schematic of the 13× myc tagging procedure

Note: The following figures outline the myc tagging protocol, which uses pADH34. Refer to the text above for a description of the minor variations in this process that are specific to TAP tagging with pADH52.

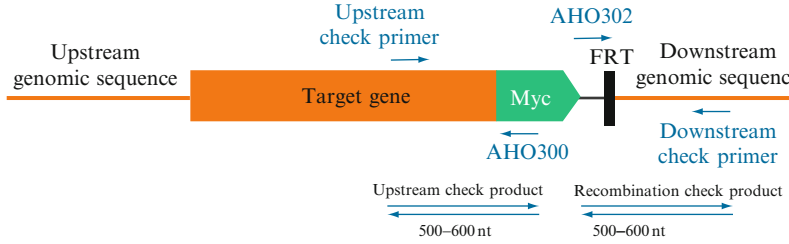
A Schematic diagram of pADH34, including the regions to which the forward and reverse knock-in PCR primers hybridize



B Schematic of the integrated knock-in cassette and locations of flank check primers



C Schematic of the tagged gene following excision of the SAT1/FLP cassette



5. *C. albicans* Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) procedures with *C. albicans* are comparable overall to those used with *S. cerevisiae* and mammalian cells, and the following protocol is based on standard ChIP methods (For example, see Lee *et al.*, 2006). We have found, however, that the methods used for cell lysis and DNA shearing are critical for performing high-resolution genome-wide ChIP (ChIP-chip) experiments with *C. albicans*. The following protocol has been used successfully, with reproducible results, to perform high-resolution ChIP-chip experiments with planktonic cultures of *C. albicans*, *Kluyveromyces lactis*, and *S. cerevisiae* (Tuch *et al.*, 2008). This protocol has also been used, with increased lysis times, to perform ChIP with *C. albicans* biofilms (Nobile *et al.*, 2009) and with both yeast and mycelial forms of *Histoplasma capsulatum* (Nguyen and Sil 2008; Webster and Sil, 2008). We also describe a rapid method for amplification of ChIP DNA samples and hybridization to high-density oligonucleotide tiling arrays.

In the previous section, we described a method for C-terminal epitope tagging in *C. albicans* that can be used to rapidly tag genes of interest for ChIP. Although the use of affinity-purified polyclonal antibodies raised against a unique peptide within a protein of interest is arguably a less disruptive method of immunoprecipitation, there are several drawbacks to such an approach. These ''peptide antibodies'' are costly, take time to produce, and often require extensive optimization for ChIP experiments. To control for cross-reactivity, which is often a problem with peptide antibodies, a viable gene deletion strain is required as a negative control, making ChIP results with essential genes much more difficult to validate. Lastly, at least two different peptides from each protein of interest should be used to raise antibodies, as it is not unusual to have one or both sets of antibodies fail completely in ChIP experiments. C-terminal epitope tagging and immunoprecipitation with commercially available, high-specificity monoclonal antibodies offers a rapid, economical, and effective method to circumvent many of these problems.

5.1. Chromatin immunoprecipitation protocol

Step 1: Culture growth and cross-linking

1. Grow 200–400 ml of cells to OD = 0.4
   Note: ~200 ml of OD = 0.4 is sufficient for one batch of lysate, which is sufficient material for as many as 10 individual ChIPs.

2. Add fresh formaldehyde (if previously opened, use within 1 month) to final concentration of 1% (stock is 37%) and cross-link 15 min at room temperature with occasional mixing.
3. Quench cross-linking by adding 2.5 *M* glycine (make fresh) to a final concentration of 125 m*M* and incubate for 5 min at RT.
4. Collect cells by centrifugation for 10 min at $1000 \times g$ in a fixed-angle centrifuge rotor.
5. Decant and resuspend pellets in 10 ml ice-cold TBS and transfer to 15 ml Falcon tubes, pellet, decant, and repeat wash 1 more time, then resuspend pellet in 2 ml ice-cold TBS. Split cell suspension to two 2 ml Sarstaedt tubes, pellet, decant, and freeze pellets in liquid nitrogen. Store at -80 °C or proceed to step 2 (skip freezing).

Step 2: Cell lysis and immunoprecipitation

1. Thaw cell pellets on ice, weigh the pellet (tare scale w/empty tube) and resuspend pellets in 700 μl ice-cold lysis buffer with protease inhibitors (Add protease inhibitors immediately prior to use at the following final concentrations: 1 m*M* PMSF, 1 m*M* benzamadine, 1 μg/ml each leupeptin, pepstatin, and bestatin; alternatively, Roche complete protease inhibitor cocktail (EDTA-free, catalog #11836170001) can be used; mix 1 Roche tablet with 10 ml lysis buffer).
2. Transfer cell suspension to a fresh 1.75-ml microfuge tube filled to the 500 μl mark with 0.5 mm glass beads.
3. Place in an Eppendorf mixer (part #5432), clamped vortex genie, or equivalent for $\sim$2 h at 4 °C.

   Note: Mixing times may vary, depending on cell type and growth conditions. For example, this technique has been used successfully with biofilms by extending the mixing time to >4 h on a vortex mixer.
4. Check cell lysis under the microscope and if $>90\%$ of cells are lysed, proceed to step 5.

   Note: Cells should appear as a mixture of "ghosts" and fragmented cell debris by phase contrast microscopy.
5. *Recover the lysate*: Invert the tubes containing lysate/beads and wipe with 70% ethanol. Allow to dry then pierce the bottom of the tube with a 26-gauge needle. Open the tube and place it (right side up) into a 5-ml falcon tube and pierce the falcon tube (above the level of the bottom of the microfuge tube) with an 18-guage needle attached to a vacuum line. The lysate should flow through to the bottom of the falcon tube (alternate: recover by centrifugation into a larger tube). Recover the lysate and transfer 300 ml to each of two fresh 1.75 ml microfuge tubes (for Bioruptor shearing) or transfer entire lysate to one fresh 1.75 ml tube (for microtip sonication).

6. Shear chromatin by sonication in a Diagenode Bioruptor^{TM} (15 min, high setting, 30 s on, 1 min off) or with a microtip sonicator (5×20 s at level 2, 100% duty cycle, with 1 min on ice between each pulse).

   Note: Shearing with a Bioruptor yields smaller fragment sizes, tighter shear distribution, and greater consistency than the tip sonication method and is highly recommended for ChIP-chip applications.
7. Pellet cell debris for 5 min at 14,000 rpm at 4 °C and transfer the supernatant (lysate) to a fresh tube.
8. Remove 50 μl of the lysate and transfer to 200 μl TE/1% SDS. This is the "input DNA" sample which can be stored at −20 °C until the end of step 3 when it is processed along with the immunoprecipitated DNA.
9. Aliquot and dilute sheared lysate according to the number of IPs to be perfomed. For each IP, use 50–500 μl of crude lysate in 500 μl (final volume) lysis buffer (with fresh protease inhibitors). The relative amounts of lysate in each IP can be equalized between strains or samples by normalizing against the mass of each cell pellet.
10. Add antibody (typically 5 μg of affinity-purified polyclonal antibody or 2 μg of monoclonal anti-myc antibody) and incubate overnight at 4 °C on a nutator.
11. The next day, add 50 μl of a 50% slurry of protein-A or protein-G Sepharose beads (washed two times with TBS and three times with lysis buffer) and incubate at least 2 h at 4 °C on a nutator.

Step 3: Recovery of immunoprecipitated DNA

1. Wash beads as follows:
   Pellet 1 min at 1000×*g* and draw off the supernatant with an 18-gauge needle on a vacuum line. Wash with the buffers indicated below for 5 min each with mixing on a nutator:
   2× with 1 ml lysis buffer
   2× with 1 ml lysis buffer w/500 m*M* final NaCl
   2× with 1 ml Wash buffer
   1× with 1 ml TE

   Note: Although Wash buffer temperatures, incubation temperatures, and incubation times can all be optimized for each antibody, we have found that ice-cold buffers and 5 min incubations at room temperature work best for most antibodies.
2. After the last wash, draw off TE and add 110 μl elution buffer, vortex, and incubate 10 min at 65 °C, mixing every 2 min.
3. Pellet 30 s at 14,000 rpm at room temperature and remove 100 μl to a fresh tube.
4. Add 150 μl TE + 0.65% SDS and vortex vigorously. Pellet and remove 150 μl and pool with previous eluate (250 μl final).
5. Incubate IP and "input DNA" samples (from step 2) for ~16 h at 65 °C.

Step 4: Cross-link reversal and DNA cleanup

1. Add 250 μl of proteinase K mix (for each sample: 238 μl TE, 2 μl 5 mg/ml glycogen, 10 μl 10 mg/ml proteinase K) and incubate 2 h at 37 °C.
   Note: Make a fresh proteinase K solution each time from lyophilized powder.
2. Add 55 μl 4 *M* LiCl and 500 μl phenol:chloroform:isoamyl alcohol (25:24:1), pH 8.0. Vortex briefly, then spin 1 min at > 10,000×*g* and remove 500 μl of the aqueous layer to a fresh tube.
   Note: AMRESCO Biotechnology Grade phenol:chloroform:isoamyl alcohol (code 0883-100 ml) has provided reliable performance in this protocol.
3. Add 1 ml ice-cold 100% ethanol and incubate at −20 °C overnight or at least 1 h at −80 °C.
4. Centrifuge 30 min at > 10,000×*g* at 4 °C and decant carefully with a 1-ml pipette.
5. Wash pellet 1× with 70% EtOH, spin 5–10 min, decant, spin briefly, and remove residual EtOH.
6. Air dry the pellets and resuspend. Use 25 μl TE for IP samples and 100 μl TE + 100 μg/ml RNaseA for input DNA samples.
7. Incubate input DNA/RNaseA solution for 1 h at 37 °C, then store at −20 °C.
   Note A: An optional DNA cleanup step could be performed on the input DNA following this step (i.e., a commercial DNA cleanup kit), however, this adds an additional variable (relative to the IP'd DNA) and may actually contribute to "spiky" data in ChIP-chip experiments. It is probably safest to leave the RNaseA in the input DNA sample and avoid any cleanup steps prior to amplification.
   Note B: Although chromatin shearing with the Bioruptor is highly reproducible (assuming cell lysis is >90%), it is advisable to monitor sheer distribution of the input DNA sample prior to proceeding with subsequent analysis of ChIP samples. Test the sheer distribution by running ~200–500 ng of purified input DNA (purify an aliquot with a DNA purification mini column) on a 2% agarose gel at ~5 V/cm. The average sheer size from the Bioruptor is typically ~200 bp, with most fragments distributed between 100 and 400 bp.

5.2. Chromatin immunoprecipitation buffers

Be sure to use autoclaved ddH_2O and baked glassware when making buffers to avoid DNA contamination. This caution is especially important for the final Wash buffers and post-elution steps.

TBS: 20 m*M* Tris/HCl (pH 7.5), 150 m*M* NaCl

Lysis buffer: 50 m*M* HEPES/KOH (pH 7.5), 140 m*M* NaCl, 1 m*M* EDTA, 1% Triton X-100, 0.1% Na-deoxycholate
Lysis buffer w/500 mM NaCl: same as above, increase total NaCl concentration to 500 m*M*
Wash buffer: 10 m*M* Tris/HCl (pH 8.0), 250 m*M* LiCl, 0.5% NP-40, 0.5% Na-deoxycholate, 1 m*M* EDTA
Elution buffer: 50 m*M* Tris/HCl (pH 8.0), 10 m*M* EDTA, 1% SDS
TE/0.67% SDS: 10 m*M* Tris/HCl (pH 8.0), 1 m*M* EDTA, 0.67% SDS
TE/1% SDS: 10 m*M* Tris/HCl (pH 8.0), 1 m*M* EDTA, 1% SDS
4 *M* LiCl
2.5 *M* glycine (fresh)
10 mg/ml proteinase K in TE (fresh)
5 mg/ml glycogen (in TE)

5.3. Strand displacement amplification of ChIP samples

The following protocol uses high concentration exo$^-$ Klenow (New England Biolabs #M0212M) and random DNA nonamers to perform strand displacement amplification of the input and IP DNA samples from ChIP experiments. Prior to amplification, input and IP DNA concentrations are normalized by dilution of the input DNA for each corresponding IP based on the qPCR values for a nonenriched locus, such as the ADE2 ORF (primers AHO294: GTTGTCAGATCATTAGAAGGGGAAG and AHO295: AAGTATCTGGGATCCTGGCA). Input and IP samples are amplified separately, in parallel, and should yield similar amounts of product following each round of amplification. Typically, three rounds of amplification are required prior to dye coupling and hybridization of the ChIP samples. If the IP DNA concentration is sufficient, Round B amplification can be omitted. Since this is a nonspecific amplification, all DNA will be amplified by this approach; all amplification steps should be performed with clean gloves, filter tips, autoclaved ddH_2O and dedicated reagents which are free of any contaminating DNA.

- *Round A (primary amplification)*:
  1. Mix:
     - 12 μl of IP sample or diluted input (diluted in TE)
       Note: equalize the input and IP samples based on qPCR values for a nonenriched locus.
     - 12 μl H_2O
     - 20 μl 2.5× SDA buffer
  2. Incubate 95 °C, 5 min then immediately transfer to an ice water bath for 5 min.
  3. Add 5 μl dNTP mix (1.25 m*M* each nucleotide).
  4. Add 1 μl 50 U/μl exo$^-$ Klenow (NEB).

5. Incubate 2 h at 37 °C with heated lid in a thermal cycler.
   Note: If needed, let the reactions sit up to ~2 h at 10 °C following amplification or add 5 μl 0.5 *M* EDTA and store at −20 °C.
6. Purify product with Zymo[25] columns (Zymo Research):
   Add at least 3 volumes of binding buffer, bind, wash one time with 200 μl binding buffer, two times with 200 μl Wash buffer, spin 1 min at 10,000×*g* to dry, and elute with 30 μl H_2O into a fresh tube.
7. Check 1.5 μl on a NanoDrop spectrophotometer (Thermo Scientific). If ≥400 ng total, skip to Round C. Otherwise continue with Round B.

- *Round B (secondary amplification)*:
  1. Mix:
     – 24 μl Round A DNA
     – 20 μl 2.5× SDA buffer
  2. Repeat steps 2–7 of Round A, but elute with 50 μl H_2O.
- *Round C (aminoallyl-dUTP incorporation and final amplification)*:

Preferred approach:

Perform 100 μl reactions with 1–2 μg total Round B DNA for each sample. Yields only ~2.5- to 3-fold amplification, but dye coupling is still very efficient.

1. Mix:
   – 1–2 μg of Round B DNA + H_2O to 48 μl total
   – 40 μl 2.5× SDA
2. Incubate 95 °C, 5 min then immediately transfer to an ice water bath for 5 min.
3. Add 10 μl 1.25 m*M* aminoallyl-dNTP mix (1:10 dilution of stock solution).
4. Add 2 μl 50 U/μl exo$^-$ Klenow.
5. Incubate 2 h at 37 °C with heated lid in a thermal cycler
   Note: If needed, let the reactions sit up to ~2 h at 10 °C following incubation or add 5 μl 0.5 *M* EDTA and store at −20 °C.
6. Purify Round C product with Zymo[25] columns: Add at least 3 volumes of binding buffer, bind, wash one time with 200 μl binding buffer, two times with 200 μl Wash buffer, spin 1 min at 10,000×*g* to dry, and elute with 50 μl H_2O into a fresh tube.
7. Check 1.5 μl on NanoDrop; the yield should be ~5 μg of total DNA per reaction.

Alternate Round C approach:

If Round B yields less than 1 μg total DNA, set up 2× 100 μl Round C reactions for each sample, using 200–400 ng of Round B DNA per tube. Perform amplification and cleanup as described for the preferred approach, but pool the two independent reactions prior to the Zymo[25] column purification.

5.4. Strand displacement amplification solutions

2.5× SDA mix: (best if made fresh, but can be kept at −20 °C for up to 1 month)
- 125 m*M* Tris–HCl, pH 7.0
- 12.5 m*M* $MgCl_2$
- 25 m*M* BME
- 750 μg/ml random DNA nonamers (dN9)

10× aminoallyl-dNTP stock solution:
- 12.5 m*M* dATP
- 12.5 m*M* dCTP
- 12.5 m*M* dGTP
- 5 m*M* dTTP
- 7.5 m*M* aa-dUTP

5.5. Dye coupling

1. Speed-vac amplified input and IP reactions from Round C to ≤9 μl each, or until dry.
2. Resuspend or QS to 9 μl with H_2O and add 1 μl of fresh 1 *M* Na bicarbonate, pH 9.0.

   Note: Prepare Na bicarbonate on the day of labeling and carefully pH using a pH meter.
3. Immediately add 1.25 μl Cy3 (input sample) or Cy5 (IP sample)

   Note: We use Amersham monoreactive dye packs (Cat. #PA23001 and PA25001). Each tube contains sufficient die for eight labeling reactions. Resuspend the dye in 10 μl DMSO and use 1.25 μl of dye per labeling reaction. If fewer than eight reactions will be performed, either decrease the volume of DMSO to use the entire tube or aliquot and speed-vac the unused dye. Store any unused dye under desiccation at 4 °C, protected from light.
4. Incubate labeling reactions for 1 h at room temperature in darkness.
5. Purify dye-coupled DNA with Zymo[25] columns (Zymo Research):

   Add 800 μl of Zymo DNA binding buffer to each sample and load onto a Zymo[25] column. Wash one time with 200 μl DNA binding buffer, two times with 200 μl Wash solution, spin 1 min at 10,000×*g* to dry, then elute with 50 μl H_2O. Check 1.5 μl on a NanoDrop spectrophotometer using the "microarray" setting to quantitate the total yield and dye-coupling efficiency (Typically > 20 picomoles of dye per microgram of DNA).
6. Proceed to array hybridization, following the array manufacturer's guidelines. Equalize the input and IP samples to 5 μg each for a 1×244 K format Agilent microarray.

Note: We have found Agilent custom oligonucleotide arrays, hybridization buffers, and Wash buffers to consistently yield high-quality data. The following hybridization protocol was adapted from the Agilent oligo aCGH/ChIP-on-Chip hybridization kit.

5.6. ChIP-chip hybridization protocol (adapted from the Agilent oligo aCGH/chip-on-chip hybridization kit)

This protocol is for competitive hybridization of amplified, dye-coupled ChIP and input DNA using Agilent 1×244 K format oligonucleotide tiling array. While a newer version of this protocol can be found on the Agilent website, we include this protocol and notes for convenience. Please follow manufacturer's guidelines for other array formats.

1. Mix 5 μg each (input and IP) sample and bring volume to 150 μl with H_2O.
   Note: Less DNA can be used; as little as 1 μg each of input and IP samples have been successfully hybridized and scanned with no significant decrease in data quality.
2. Add 50 μl of 1 mg/ml Human Cot-1 DNA (Invitrogen).
3. Add 50 μl of 10× Agilent blocking agent.
4. Add 250 μl of Agilent hybridization buffer.
5. Mix thoroughly then quick-spin to collect.
6. Incubate 3 min at 95 °C then transfer immediately to 37 °C for 30 min.
7. Spin 1 min at full speed in microcentrifuge then carefully remove 490 μl, load onto gasket slide, cover with array slide, and assemble hybridization chamber.
8. Hybridize for ~40 h at 65 °C in an Agilent microarray hybridization oven with the rotation speed set at "20."
9. Disassemble the array and wash using Agilent Wash buffers.
   a. Incubate array 5 min with mixing in Agilent oligo aCGH/ChIP-on-Chip Wash Buffer 1 at 25 °C.
   b. Incubate 5 min with mixing in Agilent oligo aCGH/ChIP-on-Chip Wash Buffer 2 at 32 °C.
   c. Incubate 1 min with mixing in acetonitrile at 25 °C.
   d. Incubate 30 s with agitation in Agilent drying and stabilization solution.
      Note: To ensure even drying, very slowly remove the slide holder such that ~10 s elapse prior to complete removal from the solution.

   Note: For disassembly, hold the microarray/gasket slide "sandwich" submerged in Wash buffer 1 while gently gripping sides of the microarray slide. Carefully pry the gasket slide off of the array by inserting the tip of a plastic forceps between the outer edge of the two slides and lightly twisting the forceps. The gasket slide will fall away, while the array should remain in your hands. Be sure to avoid any contact with the printed array surface.

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