Chapter 2

Constructing Synthetic Microbial Communities to Explore the Ecology and Evolution of Symbiosis

Adam James Waite and Wenying Shou

Abstract

Synthetically engineered microbial communities based on model organisms provide a simplified model of their naturally occurring counterparts while still retaining essential features of living organisms. The degree of control afforded by this approach has been critical in understanding how similar types of natural communities might have persisted and evolved. Here, we first discuss important considerations when designing a synthetically engineered system. Then, we describe the steps required to create a two-partner cooperative system based on the yeast *Saccharomyces cerevisiae*.

Key words Evolution, Ecology, Mutualism, Cooperation, Synthetic biology, *S. cerevisiae*

1 Introduction

From mediating biogeochemical cycles [1] to influencing human health [2] and disease [3], microbial communities impact all aspects of life on earth. However, the complexity of microbial communities and the difficulty in isolating and culturing microbes [4] pose serious challenges for decoding cell–cell and cell–environment interactions. Moreover, the evolutionary histories of microbial communities are difficult to retrace. Alternatively, communities of model organisms engineered to engage in defined interactions can be deployed to address fundamental questions in ecology and evolution, such as how species coexist and coevolve [5]. In this chapter, we discuss several considerations when designing a synthetic community, using the construction of a two-partner cooperative yeast system as an example. Then, we describe the methodology in detail.

The initial consideration is the choice of organisms. Genetic tractability and short generation times facilitate strain construction and experimentation, as well as the discovery and interpretation of mutations during experimental evolution. Thus, well-studied model organisms with reference genome sequences such as *Escherichia coli*

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or *Saccharomyces cerevisiae* are ideal, although other species have also been used [5]. While each model organism has its advantages and disadvantages, we will use *S. cerevisiae* to highlight principles that are applicable to any synthetically engineered community.

Sexual recombination is both a help and a hindrance to synthetically engineered communities. On the one hand, sexual recombination can radically simplify strain construction by allowing genetic shuffling. For example, when a strain with genotype *AB* is crossed with a strain of genotype *ab*, recombinant strains *Ab* and *aB* can be generated. On the other hand, distinct populations in an engineered community should remain genetically insulated from one another and therefore should not be allowed to mate. An advantage of using *S. cerevisiae* is its ability to undergo both sexual and asexual reproduction. Haploid yeast is particularly suitable for evolution experiments since phenotypes arising from recessive mutations are immediately apparent. Haploid yeast can reproduce asexually as either of the two mating types, "a" or "α." Two cells of opposite mating type can mate to produce an a/α diploid, which can reproduce either asexually as diploids or sexually to form haploids. The final strains for an engineered community should always be the same mating type to prevent sexual recombination. However, haploid yeast switch mating types spontaneously at very low frequency even when the gene required for mating-type switching (*HO*) is defective (which is the case for all commonly used laboratory strains). Thus, we have used *MAT***a** cells in which the *STE3* gene encoding the receptor for *MAT***a** mating pheromone [6] is deleted. Thus, if a *MAT***a** *ste3* cell switches mating type to *MAT***α** *ste3*, it will fail to initiate the mating process.

Ideally, all strains should be derived from an isogenic background so that the only mutations are the ones defined by the researcher. We have used the strain S288C, which is one of the common laboratory strains, and the wild vineyard isolate RM11-1a (hereafter referred to as "RM11"). While S288C has its genome fully annotated and easily accessible [7], it has a tendency to produce mitochondrially deficient "petite" cells [8], which are prone to nuclear genome instability [9] and could potentially interfere with evolution experiments. RM11 (genome sequence available at http://www.broadinstitute.org/annotation/genome/saccharomyces_cerevisiae/) grows faster than S288C and produces very few petites, but haploid daughter cells do not separate well from their mothers unless the RM-11 *AMN1* allele is replaced by the *AMN1* allele from S288C [10]. Genetic manipulation in RM-11 is more difficult due to its lower transformation efficiency compared to S288C.

A major advantage of using model organisms is that they are genetically modifiable. Foreign DNA can be transformed into yeast as autonomously replicating and segregating circular plasmids or as

linear DNA if genomic integration is desired. Integration is more stable than using a plasmid. Different populations can be marked with, for example, different antibiotic resistances or fluorescent proteins. Currently, at least six dominant antibiotic resistance genes are in wide use with *S. cerevisiae*: *kan*, which confers resistance to geneticin a.k.a. G418; *hph*, which confers resistance to hygromycin B; *nat*, which confers resistance to nourseothricin (sold as clonNAT by Werner BioAgents); *pat*, which confers resistance to phosphinothricin; *ble*, which confers resistance to phleomycin; and *AUR1-C*, which confers resistance to Aureobasidin A (AbA). All of these genes are available on plasmids [11–13]. Our lab has used G418, hygromycin B, clonNAT, and AbA resistance markers. Using antibiotic resistance to select a subpopulation from a co-culture is especially useful if the subpopulation is very rare, as tens of millions of cells can be assayed on a single plate. However, plating on media supplemented with different drugs to determine subpopulation abundance is time-delayed since it takes at least 1 day for colonies to grow up. It is also of low throughput due to the small number (hundreds) of individual colonies that can be counted on a plate. In contrast, fluorescently tagged strains can be distinguished using flow cytometry, which allows tens of thousands of cells to be counted in less than a minute. While fluorescence-activated cell sorting (FACS) can also be used to isolate subpopulations, it requires an expensive instrument and is less efficient when isolating very rare subpopulations. A large number of fluorescent proteins are available [14], although not all of them are bright and/or resolvable from one another using standard filter sets. In addition, the correct folding of fluorescent proteins requires oxygen [15] and is therefore incompatible with strict anaerobes. We have C-terminally tagged (Fig. 1) the highly abundant proteins *FBA1* or *MET6* with different fluorescent proteins in yeast. Using the appropriate combination of lasers and filter sets (Table 1), we can resolve mixtures of cells expressing five different fluorescent proteins: CFP, GFP, YFP, mOrange, and mCherry as well as the far-red nucleic acid dye TO-PRO-3 (Invitrogen) which can be used to stain dead cells. Plasmids containing different combinations of fluorescent proteins and selectable markers (i.e., genes for nutrient biosynthesis or antibiotic resistance) [16] are readily available from EUROSCARF (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/).

Since deleting or inserting genes will usually be accomplished by transformations (Fig. 1), which require selectable markers, it is convenient to be able to reuse these markers. Plasmids containing *kanMX* [17] and *ble* [18] flanked by loxP sites are available for this purpose (for a comprehensive list of plasmids with removable markers, *see* ref. 19). In the presence of Cre recombinase, the two loxP sites recombine, removing the intervening marker and allowing for its reuse in another round of manipulation. Our lab has

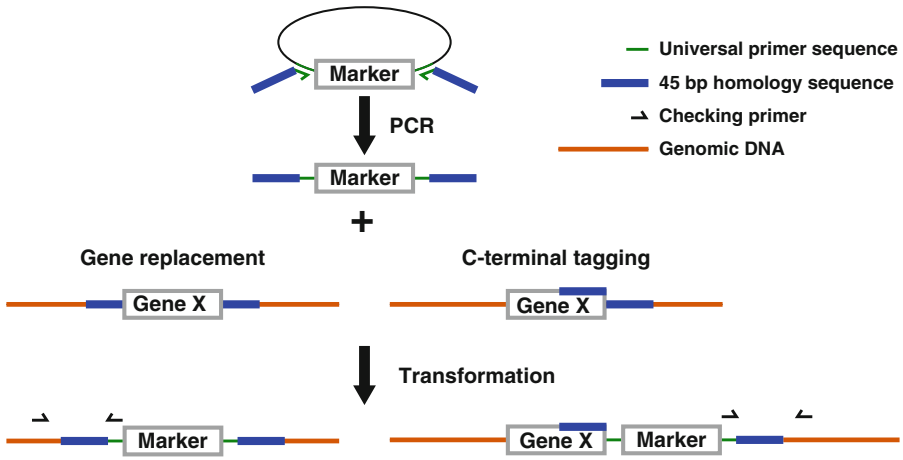

Fig. 1 Schematic of gene replacement and C-terminal tagging. In both cases, the selectable "marker" is PCR amplified off a plasmid using a pair of hybrid primers. The 3′ end of the primers contains sequences specific to the plasmid (*thin green lines*) in a region ideally identical among a family of marker plasmids to achieve flexibility. The 5′ end of the primers is 45 bp homologous to yeast genomic DNA (*thick blue lines*). For gene replacement, the forward homology is to the region immediately 5′ of the start codon of the open reading frame (ORF) of the gene of interest ("Gene X"), while the reverse homology is the reverse complement of the region immediately 3′ of the stop codon of the ORF. For C-terminal tagging, the reverse homology is the same as for gene replacement, while the forward homology is the 45 bp leading up to but not including the stop codon of the ORF. After transformation, checking primers (*thin black arrow lines*) are used in colony PCR to verify proper integration of the DNA fragment. One primer has homology to the plasmid sequence, while the other has homology specific to the region outside of the 45 bp homology used for integration. For C-terminal tagging, the depicted location of checking primers is preferred, as it results in a shorter (and therefore easier to amplify) PCR product

Table 1
Lasers and filter sets used to simultaneously resolve five fluorescent proteins and one fluorescent dye

Laser (nm)	Filter[a]	Fluorophore
405	450/50	CFP
488	505/10 530/30	GFP YFP/citrine
561	590/20 615/25	mOrange mCherry
639	660/16	TO-PRO-3

[a]The number before the slash indicates the center wavelength in nanometers; the number after the slash is the total bandwidth passed by the filter. For example, "450/50" indicates a filter that passes wavelengths from 425 to 475 nm

modified these plasmids to contain *nat* (WSB116) and *hph* (WSB117). We generally do not use auxotrophy (the inability to synthesize an essential metabolite) to mark strains except when both selection and counterselection are required. For example,

integration of the loxP–drug–loxP cassette could be carried out in a *ura3* strain so that transformation of a *URA3*-marked plasmid containing the Cre recombinase can be selected for. After induction of Cre expression and removal of the drug marker, the *URA3* plasmid can then be counterselected using 5-FOA, which only allows survival of cells without the plasmid [20]. The uracil auxotrophy may then need to be removed via genetic crosses. Alternatively, Cre expression plasmids containing antibiotic markers are also available [19].

Once the populations have been marked, interactions between populations can be defined. Obviously, the possibilities are practically limitless, and the specifics must be left to the researcher. We chose to base our two-partner cooperative system on complementary nutrient exchange [21].

In yeast, all of the manipulations described above can be achieved through a small set of well-known methods [19]. These include (1) transformation to insert or remove genetic material; (2) colony PCR, a simple and rapid way to check whether transformation was successful; and (3) mating, sporulation, tetrad dissection, and genotyping, which allows genetic features present in two different strains to be recombined into one strain. Below we describe our current protocols for each of these methods.

2 Materials

2.1 Components for Genetic Manipulation

1. Plasmids containing genes encoding fluorescent proteins and/or selectable markers.
2. Antibiotics: 1,000× stock G418 (200 mg/ml), 500x stock hygromycin B (100 mg/ml), 1,000× stock clonNAT (100 mg/ml), 1,000× stock AbA (0.5 mg/ml) (*see* **Note 1**).
3. YPD: 10 g/l Bacto-yeast extract, 20 g/l Bacto-peptone, and bring to volume with diH_2O to final 950 ml/l for later glucose supplement. Add 20 g/l Bacto-agar (*see* **Note 2**) if making plates. Add a magnetic stir bar, and autoclave. Using a flame to ensure sterility, add 50 ml 40 % glucose per liter of medium and the appropriate antibiotic, if necessary. Stir to mix. If multiple liters of agar medium are prepared, they may be kept at 50 °C water bath to prevent solidification of the agar.
4. SD: For liquid media, add 6.7 g/l Difco™ yeast nitrogen base (YNB) with ammonium sulfate and without amino acids and 20 g/l glucose, and bring to volume with diH_2O. Sterilize using 0.22 μm filter. For plates, add 6.7 g/l YNB, 20 g/l Bacto-agar, and a stir bar, and add diH_2O to 950 ml/l. Autoclave. Using sterile technique, add supplements as necessary. Amino acid and nucleobase supplements can be mixed in appropriate proportions [19] in their powder forms in a

sterilized blender and stored at room temperature. Powdered supplements can be weighed and directly added to the media. Finally, add 50 ml 40 % glucose. Use the glucose to wash down any residual supplement powder adhering to the vessel wall and stir.

5. 50 % PEG 2000 (*see* **Note 3**): Dissolve 100 g PEG in 100 ml diH_2O. Bring to 200 ml with diH_2O. Sterile filter.
6. 1 M LiAc: 102 g/l Lithium acetate dihydrate (102.02 g/mol). Sterile filter.
7. 5 mg/ml Sheared salmon sperm DNA (SS-DNA) [22].
8. Autoclaved water, tubes, and tips.

2.2 Components for Mating, Sporulation, and Tetrad Dissection

1. Sporulation media: 3 g/l potassium acetate, 0.2 g/l raffinose, bring to final volume with diH_2O. Autoclave.
2. SCE buffer: 1 M D-sorbitol, 0.1 M sodium citrate (*see* **Note 4**), 60 mM EDTA. Adjust pH to 7.0 with 38 % HCl (*see* **Note 5**). Autoclave using a 20′ sterilization cycle, and remove promptly (*see* **Note 6**).
3. Zymolyase 20T: 30 mg zymolyase 20T dissolved in 10 ml SCE (*see* **Note 7**).

3 Methods

3.1 Primer Design and Amplification for Gene Tagging or Replacement

1. Design primers: For gene replacement (Fig. 1), the forward primer should contain 45 base pairs (bp) of homology to the genomic region immediately upstream of the "ATG" codon of the open reading frame (ORF) of the gene to be knocked out, followed by the sequence for the universal forward adapter appropriate for the particular set of plasmids being used. The reverse primer should contain the reverse complement to the 45 bp including and immediately downstream of the stop codon of the target ORF, followed by the reverse complement to the universal reverse adapter appropriate for the plasmid set. For C-terminal tagging (Fig. 1), the reverse primer is designed as in gene replacement, and the forward primer should contain 45 bp homology to the sequence just 5′ of the stop codon of the gene of interest.
2. PCR amplify cassette (antibiotic resistance for gene replacement or fluorescent protein plus selectable marker for C-terminal tagging) off a plasmid (*see* **Note 8**). Check the length of the PCR product using gel electrophoresis.

3.2 Transformation (See Note 9)

1. Inoculate 5 ml YPD culture per transformation and shake at 30 °C until the cell density is between 3×10^6 and 2×10^7 cells/ml (*see* **Note 10**).

2. Prepare a boiling water bath for the SS-DNA. While the water is warming up, harvest the culture in a sterile 50 ml centrifuge tube at 425×*g* for 2 min.
3. Pour off the YPD medium, and resuspend the cells in 25 ml of sterile water and centrifuge again.
4. Pour off the water, resuspend the cells in 100 μL 0.1 M LiAc, and transfer the suspension to a 1.5 ml microfuge tube.
5. Pellet the cells at top speed for 15 s, and remove supernatant with a micropipette.
6. Resuspend the cells in about 43 μl of 0.1 M LiAc to a final volume of 50 μl (2×10^9 cells/ml) per transformation.
7. By now the water bath should be boiling. Boil SS-DNA (with cap lock on) for 5 min, and then quickly transfer it to ice (*see* **Note 11**). Vortex briefly to speed up cooling, and then keep on ice.
8. Vortex the cell suspension and pipette 50 μl into 1.5 ml tubes. Pellet the cells at top speed for 15 s, and remove supernatant with a pipette. Vortex to loosen up the pellet.
9. Prepare the transformation mixture (TRAFO) master mix (1.2× the total volume required so that pipetting errors can be accommodated) and keep on ice (*see* **Note 12**): 240 μl 50 % w/v PEG (*see* **Note 3**), 36 μl 1.0 M LiAc, 20 μl 5 mg/ml SS-DNA, and 64-*x* μl sterile dH_2O per transformation, where *x* μl is the volume of DNA to be added (*see* **Note 13**). Vortex until completely homogenous (*see* **Note 14**).
10. Add 360-*x* μl TRAFO to each cell pellet (*see* **Note 15**), and mix well by pipetting up and down (*see* **Note 16**). Add *x* μl DNA, and mix well again by vortexing or pipetting.
11. Heat shock cells by placing the tubes in a 42 °C water bath for 40 min (*see* **Note 17**). Mix by inverting every ~10 min.
12. Centrifuge at 3,824×*g* for 15 s, and remove TRAFO with a pipette or simply by decanting. Wash cells by resuspending the pellet in 1 ml YPD. Spin again at 3,824×*g* for 15 s (*see* **Note 18**). Remove YPD, and pipette 1 ml fresh YPD into each tube. Resuspend the pellet by pipetting it up and down gently.
13. If selection is on complementation of nutrient auxotrophy, cells can be directly plated on selective medium. If selection is on drug resistance, cells need to be incubated for ~2–3 h at 30 °C in 1 ml YPD to express the resistance gene before being plated.
14. To plate, first centrifuge at 3,824×*g* for 15 s. Discard 700 μl of the supernatant. Resuspend cells in the remaining 300 μl and plate on 80 % of the surface. Use a sterile toothpick to streak from the plated area to the empty area to maximize the chance of obtaining single colonies.
15. Incubate at 30 °C. Colonies should be visible in 2–3 days.

3.3 Colony PCR (See Note 19)

1. Design primers: One primer should be specific to the cassette used for transformation (for example, the universal primer sequence). The other primer should be outside the 45 bp homology region used for integration (Fig. 1).
2. Using a sterile pipette tip, pick about half of a normal-sized (~1.2 mm diameter) colony without touching the agar beneath it. For S288C, place directly into 15 µl sterile water, vortex, and use 1 µl in a 20 µl PCR (*see* **Note 20**). For RM11, transfer the cells to 15 µl 0.25 % SDS (*see* **Note 21**). Vortex for 30 s, spin at top speed (~17,949 × *g* in a small centrifuge) for 1 min, and use 1 µl of the supernatant for a 20 µl PCR. This PCR mix must contain a final concentration of 5 % Triton X-100 to neutralize the protein-denaturing SDS [23]. An alternative, and more reliable, method uses LiAc and SDS to lyse cells and requires ethanol precipitation prior to PCR (*see* **Note 19**, ref. 22). Specifically, cells are suspended in 100 µl 200 mM LiAc and 1 % SDS solution and incubated at 70 °C for 15 min. 300 µl 96 % ethanol is added to precipitate DNA. After brief vortexing, DNA is collected by centrifugation at 15,000 × *g* for 3 min. Precipitated DNA is dissolved in 100 µl TE (10 mM Tris–HCl, 1 mM disodium EDTA, pH 8.0). After spinning cell debris down at 15,000 × *g* for 1 min, 1 µl supernatant is used for PCR.

3.4 Mating and Diploid Isolation

1. Grow up a small patch of each haploid to be crossed towards the top of a YPD plate (*see* **Note 22**).
2. Use a sterile toothpick to transfer a tiny amount of one strain to an empty region of a YPD plate (*see* **Note 23**), making a small spot (a few millimeters in diameter). With a fresh toothpick, transfer a similarly tiny amount of the other strain to the same spot and mix with the toothpick.
3. Incubate for 3.5 h (S288C) or 2.5 h (RM11) at 30 °C (*see* **Note 24**).
4. Using a toothpick, touch the mixed spot and streak down about a centimeter. Without re-touching the spot, make similar streaks to the left and right of the original streak. This gives three different dilutions of cells on the plate.
5. Use a yeast dissection microscope [19] to isolate diploids. The cytoplasmic "bridge" between mated cells and a small bud at its center is indicative of recently mated diploids.

3.5 Sporulation, Tetrad Dissection, and Genotyping

1. Pre-sporulate by patching onto YPD and incubating at 30 °C for ~10 h until a thin film of cells is formed (*see* **Note 25**).
2. Inoculate 2 ml sporulation media with about a match head's worth of cells. Incubate at room temperature on a rotator for 2–5 days. Check for tetrads using a light microscope using a 20–40× magnification objective [19].

3. Wash cells with 1 ml sterile water and resuspend in 1 ml sterile water. Store at 4 °C.
4. When ready to dissect, spin down 20 μl of sporulated cells and carefully remove supernatant with a pipette. Add 20 μl SCE, and vortex to resuspend cells. If using RM11, sonicate for 1 min.
5. Add 4 μl zymolyase 20T, and mix by pipetting up and down (*see* **Note 26**). Incubate at room temperature for 20 min (S288C) or 2 h (RM11) (*see* **Note 27**).
6. Gently (to avoid breaking up tetrads) pipette up digested cell suspension and spot onto the top portion of the center strip of a YPD plate. Tilt plate down so that the liquid rolls down the central strip, stopping before the liquid touches the plate wall. Let dry.
7. Use a yeast dissection microscope to separate tetrads into individual spores. Create a grid with a maximum of two tetrads per row [19], one to the left and one to the right of the central strip.
8. Incubate overnight at 30 °C. Use a flame-sterilized scalpel to remove the strip of cells from the middle of the plate (*see* **Note 28**) and return to incubator.
9. Once the spores have germinated and grown into colonies, replica plate onto the appropriate selective media(s). Note that a single genetic locus should, under most circumstances, segregate 2:2 [19]. For instance, two spores will be *MAT*a and two will be *MAT*α. Exceptions can be caused by, for example, gene conversion or traits that are mediated by heritable materials transmissible through the cytoplasm, such as mitochondria.
10. Mating types can be tested using a pair of mating-type testers, one of each mating type. If the entire collection of spores are auxotrophic because of mutations in a set of genes, then the testers should be auxotrophic due to mutation in a different gene to ensure that the resulting diploids are prototrophic. In this case, spread ~200 μl of a saturated culture of each tester strain on its own YPD plate and let dry. Then, replica plate the target strains onto each plate using a sterile velvet. After half a day, replica each plate onto an SD plate. Growth will only occur if the strains are able to mate. If spores are prototrophic, they need to be separately mated to each of the two tester strains, as described above for diploid isolation. Many crosses can be set up on one YPD plate. The mating type of a strain is revealed by the presence or the absence of fused diploids. If this method is necessary, it is better to narrow down the number of strains to be tested based on other markers first.

4 Notes

1. Prepare stocks in deionized (di) H_2O and sterile filter (except for AbA, which should be made in ethanol and needs no sterile filtration) and keep at −20 °C.
2. Not all sources of YNB and agar are appropriate for use with yeast. For example, we have found that YNB and agar obtained from BD biosciences work better than those obtained from USA Scientific.
3. The size of PEG is important. Transformation efficiency for RM11 is very low if PEG 3500 is used. We use PEG3500 to transform S288C, although PEG2000 should work as well.
4. NaH_2PO_4 can be used instead.
5. About 0.5 ml when making a final volume of 500 ml.
6. Ensure that the color of solution did not change during the autoclave process.
7. Freeze down 1 ml aliquots at −20 °C. To use, thaw one tube and make smaller aliquots. Store one at 4 °C for up to 2 months, and freeze the rest for later use.
8. Always use a high-fidelity polymerase for PCR to minimize the possibility of introducing mutations into the amplified fragment. Here is a specific recipe for C-terminally tagging *FBA1* using the pKT plasmids [16] and KOD polymerase (EMD Millipore): 0.5 μl miniprep DNA (~100 ng), 5 μl 10x buffer, 4 μl 25 mM $MgSO_4$, 5 μl 8 mM dNTPs, 0.5 μl primer WSO178 (50 μM), 0.5 μl primer WSO179 (50 μM), 0.5 μl KOD polymerase, 34 μl water (molecular biology grade). WSO178 sequence: 5′-AAGATCACCAAGTCTTTGGAAACTTTCC G T A C C A C T A A C A C T T T A g g t g a c g g t g c t g g t t ta-3′. WSO179 sequence: 5′-GATTCAATACTCATTAAAAA ACTATATCAATTAATTTGAATTAACtcgatgaattcgagctcg-3′. The 45 bp homology sequence is uppercase; the universal primer sequence is lowercase. PCR settings: 94 °C for 2 min, 30 cycles of {94 °C for 30 s, 55 °C for 30 s, and 70 °C for 3 min}, and then 70 °C for 10 min. When *nat* is the template, it is essential to add DMSO to a final concentration of 5 % [11].
9. Transformation is mutagenic. It is therefore best to transform diploids and then sporulate, since one round of meiotic segregation reduces the probability of obtaining an undesired mutation by 50 %. If multiple haploids of the desired genotype derived from the same diploid behave similarly, then background mutations are unlikely to be important. Alternatively, our lab has found that Illumina deep sequencing can reveal the presence of mutations caused by transformation.
10. To ensure that cultures are in exponential phase when it is time to transform, pilot growth experiments may be helpful. Wild-type RM11 grows very rapidly. If the density is $>5 \times 10^7$

cells/ml, dilute to allow the cells to complete at least two divisions in unsaturated conditions. Transformation efficiency remains constant for 3–4 cell divisions.

11. Keep small aliquots of SS-DNA to limit the number of freeze–thaw cycles. Keep on ice when out of the freezer.
12. Keeping the TRAFO master mix on ice is crucial for high efficiency.
13. Use >100 ng DNA. In general, more DNA yields more transformants, but this relationship will likely saturate at some point.
14. This can be visually confirmed by ensuring that no visible "strands" are present in the mixture.
15. TRAFO is very viscous, so pipette slowly to ensure that the correct volume is transferred.
16. Mixing well ensures that the SS-DNA effectively blocks non-specific DNA binding.
17. Adjusting the amount of time for heat shock may be necessary to achieve maximum efficiency. However, 40 min works well for S288C and RM11.
18. Be as gentle as possible at this step, as the cells are very fragile.
19. Colony PCR is quick but can be unreliable. When it fails to work, we have found that a quick DNA extraction before PCR gives reliable results [23].
20. The cell suspension should be turbid.
21. Unlike S288C, boiling of RM11 cells does not provide good template for PCR, although we do not know why. Using detergent effectively lyses the cells and releases their DNA into solution.
22. This can be done for as little as 3 h.
23. The amount should be small enough to not leave a visible film on the plate after transfer.
24. Different strains may require more or less time. Cells should show visible film of growth by this time.
25. Overgrowth will lead to a reduction in sporulation efficiency. However, a suitable number of tetrads should be present even after 12–14 h of pre-sporulation.
26. Vortexing can oxidize the zymolyase.
27. The four spores form a three-dimensional, tetrahedral shape if the ascus wall is undigested. After sufficient digestion, the four spores will have a flat, diamond shape. Underdigestion of the ascus wall will make it difficult to separate the individual spores. Overdigestion will result in tetrads that break apart easily, increasing the chances that four spores in the correct diamond shape are not products of the same meiosis. Overdigestion can also reduce spore viability.
28. Otherwise, growth of this strip will slow down the growth of the haploids nearest to it.

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