Video Article

Transformation of Probiotic Yeast and Their Recovery from Gastrointestinal Immune Tissues Following Oral Gavage in Mice

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Abstract

Development of recombinant oral therapy would allow for more direct targeting of the mucosal immune system and improve the ability to combat gastrointestinal disorders. Adapting probiotic yeast in particular for this approach carries several advantages. These strains have not only the potential to synthesize a wide variety of complex heterologous proteins but are also capable of surviving and protecting those proteins during transit through the intestine. Critically, however, this approach requires expertise in many diverse laboratory techniques not typically used in tandem. Furthermore, although individual protocols for yeast transformation are well characterized for commonly used laboratory strains, emphasis is placed here on alternative approaches and the importance of optimizing transformation for less well characterized probiotic strains. Detailing these methods will help facilitate discussion as to the best approaches for testing probiotic yeast as oral drug delivery vehicles and indeed serve to advance the development of this novel strategy for gastrointestinal therapy.

Video Link

The video component of this article can be found at <http://www.jove.com/video/53453/>

Introduction

Probiotic microorganisms are an intriguing potential means of efficiently and economically delivering heterologous proteins to the gastrointestinal tract. These organisms are capable of surviving passage through the gastrointestinal tract yet do not colonize it¹, enabling controlled dosing and limiting exposure to the drug expressed. Furthermore, the ability to easily engineer these organisms to produce heterologous protein on a large scale renders them an economical alternative to synthetic delivery particles. However, development of such an approach, as recently demonstrated using an auxotrophic strain of the probiotic yeast *Saccharomyces boulardii*², requires knowledge of laboratory techniques not traditionally combined within a given study, ranging from yeast and molecular biology to animal handling techniques and immunological methods. Thus although the individual procedures described herein are not in themselves novel laboratory protocols, the goal of this manuscript is to present a unified introduction to techniques needed for experimental testing of probiotic yeast as drug delivery vehicles to the murine gastrointestinal tract. Provided is a compilation of essential protocols for: 1) generation of auxotrophic mutant strains of yeast that can easily be genetically manipulated; 2) transformation of yeast cultures to express heterologous protein; 3) administration of recombinant yeast to the intestine via oral gavage; and 4) recovery of viable recombinant probiotic yeast from the murine intestine and assessment of their heterologous protein expression.

First, although numerous positive and negative selection methods exist for the manipulation of yeast species, negative selection such as through the use of auxotrophic markers increases both the efficiency and ease with which yeast can be transformed and selected. Positive selection of transformants using antibiotics, in contrast, significantly increases the cost of yeast manipulation. Furthermore, selection of yeast on antibiotic-containing solid media can allow for increased growth of untransformed background colonies relative to selection of auxotrophic yeast on synthetic drop out solid media (unpublished observations). Auxotrophic yeast is strains which lack enzymes critical for the synthesis of essential amino acids or uracil. Such yeast can grow only if supplemented with the missing metabolite or metabolic gene, thus enabling negative selection when yeast is plated onto synthetic drop out media that lacks the essential metabolite. Many commonly used *Saccharomyces cerevisiae* laboratory strains are in fact already auxotrophic mutants³. Industrial, clinical, and probiotic yeast strains, however, are typically prototrophic with the ability to synthesize all required nutrients. To enable more efficient genetic manipulation of such yeast, auxotrophic genes can be selectively targeted to generate strains that can be selected without antibiotics. Specific targeting of auxotrophic marker genes can be achieved through PCR-mediated gene disruption relying on homologous recombination or more recently through CRISPR/Cas9 targeting⁴⁻⁶. Alternatively, UV mutagenesis can quickly generate auxotrophic mutants even in yeast strains for which transformation with multiple plasmids is technically difficult⁷. While PCR targeting and CRISPR/Cas9 have been described extensively elsewhere, presented in part one of this manuscript is a detailed protocol describing a UV mutagenesis approach to create auxotrophic strains that will allow for negative selection rather than positive antibiotic selection of yeast transformants.

The next necessary step in the use of such auxotrophic strains for oral delivery of heterologous protein is yeast transformation with plasmid DNA. Since the first successful transformation of yeast spheroplasts reported for *Saccharomyces cerevisiae* in 1978⁸, numerous modifications have been characterized to increase the efficiency and ease with which yeast species can be genetically modified. Use of electroporation for the successful transformation of DNA into *S. cerevisiae* was first described in 1985⁹ and has since been improved via the addition of 1 M sorbitol incubation to osmotically support cells¹⁰. Electroporation efficiency has furthermore been shown to depend on the yeast species and strain, cell number and phase of growth, electroporation volume, field strength, and specific buffers¹¹. Lithium acetate (LiOAc) transformation, originally described by Ito *et al.*¹², is among the most commonly used transformation protocols as it requires no special equipment. Additional analyses showed that the efficiency of LiOAc yeast transformation greatly increases when cells are collected in mid-log phase of growth and are heat shocked in the presence of polyethylene glycol (PEG) and DNA at 42 °C¹². Incubation of whole intact yeast with PEG is essential for efficient transformation, possibly through improving attachment of DNA to the cell membrane as well as via other effects on the membrane¹³. Lithium itself also increases the permeability of intact cells¹⁴. Although most laboratory *S. cerevisiae* strains can easily be transformed using LiOAc transformation³, other yeast species may be more efficiently transformed using alternative protocols. *Pichia pastoris*, for example, is most efficiently transformed via electroporation rather than LiOAc transformation¹³. It is crucial, therefore, to test multiple methods of transformation and to optimize incubation periods and reagent concentrations when attempting to genetically modify an uncharacterized yeast strain. This manuscript thus describes both LiOAc transformation and electroporation as techniques for the transformation of auxotrophic mutant and wild type *S. boulardii*. Interested readers are directed to recent reviews for thorough descriptions of the evolution of yeast transformation, alternative protocols, and further discussions of possible mechanisms of action^{13,15}. Transformation of yeast with plasmid encoding an easily detectable protein is furthermore essential for downstream testing in order to ensure proper expression and function of heterologous protein. Myriad different proteins may be selected depending on the ultimate purpose of the therapeutic study and the antibodies available for protein detection by immunoblotting, ELISA, and other techniques. Protocols for these techniques have been thoroughly described elsewhere^{16,17}, and can be used to determine levels of heterologous protein production from transformed yeast by comparison to standard curves. For purposes of demonstration and to show successful production of a very commonly used protein in yeast biology, this manuscript presents transformation with plasmid encoding green fluorescent protein (GFP), which allows for subsequent detection using fluorescence microscopy.

Equally important to the production of probiotic organisms that express heterologous protein is the proper administration and detection of these microorganisms within gastrointestinal tissues, as described in parts three and four. Administration of recombinant yeast via oral gavage allows for delivery of controlled quantities of yeast directly into the stomach, from which C57BL/6 mice are naturally incapable of vomiting¹⁸. However, improper animal handling and gavage can lead to esophageal damage and perforation, gastric perforation, tracheal administration, and aspiration pneumonia^{19,20}. Poor technique and inexperience can furthermore increase variability in murine immune responses and experimental results, which have been attributed to animal stress upon oral gavage^{21,22}. Practice in the proper technique can thus not only attenuate animal discomfort, but can also increase precision of experimental results. This manuscript describes and demonstrates animal handling and oral gavage for the administration of controlled doses of recombinant yeast.

Finally, it is vital to confirm successful delivery of recombinant yeast by analyzing lymphoid tissues for the presence of yeast and heterologous protein. The gastrointestinal immune tissues which can most easily and predictably be examined for the presence of yeast are the Peyer's patches. Peyer's patches are secondary lymphoid organs along the small intestine that are key sites of mucosal immune response induction²³. Antigens from the lumen are transferred transcellularly through microfold (M) cells in the epithelium and are released into the Peyer's patches, thus exposing enclosed antigen presenting cells to intestinal luminal contents. Although particle uptake across the intestinal epithelium can also be achieved by goblet cells, these cells have been shown to only take up particles less than 0.02 µm in diameter²⁴. Transepithelial dendrites extended from CD103⁺ dendritic cells (DC) also take up small particles from the intestinal lumen²⁵; however, there are currently no reports demonstrating that CD103⁺ DCs take up particles larger than bacteria. Thus, intact probiotic yeast, of average size between 3-6 µm in diameter, are most likely to be taken up by M cells and transferred to the Peyer's patches. Described here is a protocol for collection and screening of Peyer's patches for viable recombinant yeast, although this procedure can also be easily adapted for evaluating uptake of probiotic bacteria.

In summary, assessing recombinant probiotic yeast for the delivery of therapeutic proteins to the intestine requires proficiency in laboratory techniques spanning molecular biology to animal handling and immunology. Presented here are protocols for 1) the generation and screening of auxotrophic yeast strains which can be easily negatively selected without antibiotics, 2) alternative protocols to transform yeast and enable expression of heterologous protein, 3) demonstrations of proper animal handling techniques and oral gavage for intragastric delivery of recombinant yeast, and 4) protocols for Peyer's patch dissection and screening for viable recombinant yeast and functional heterologous protein. Combined, these protocols will allow for the generation and testing of a probiotic yeast strain capable of delivering heterologous therapeutic protein to the gastrointestinal tract.

Protocol

1. UV Mutagenesis to Generate Auxotrophic Yeast Strains

1. Generate survival curves to determine needed doses of UV irradiation
 1. Prepare YPD (yeast extract peptone dextrose) media and other reagents listed in **Table 1** according to standard procedures²⁶ and inoculate single colonies into 5-10 ml of YPD media. Incubate cultures on a roller drum at 30 °C O/N to saturation for at least 8 hr.
 2. Determine the cell concentration of O/N cultures using a spectrophotometer by diluting cells 1:10 in water in a plastic cuvette. Dilute cells to a concentration of 10⁷ cells/ml in 20 ml sterile distilled water.
 3. Pour diluted cells into a sterile plastic Petri dish and, with the lid removed, place the plate 14 cm below a UV bulb.
 4. Expose cells to serial 5,000 µJ and 10,000 µJ doses of UV irradiation, extracting 500 µl of cells following each increment such that cells are sampled after exposure to 0 µJ, 5,000 µJ, 10,000 µJ, 15,000 µJ, 20,000 µJ, 25,000 µJ, 30,000 µJ, 40,000 µJ, and 50,000 µJ of UV irradiation. Transfer extracted cell samples to sterile 1.5 ml tubes and serially dilute at 1:10 increments in sterile water.
 5. Pellet cells in each dilution by centrifugation in a microcentrifuge at 16,000 x g for 1 min. Aspirate supernatant and resuspend in a 100 µl volume of sterile water appropriate for plating yeast cells. Pipette the full volume of resuspended cells onto plates containing YPD solid media and use a sterile spreader to evenly distribute cells across each plate.

6. Wrap plate edges in Parafilm to prevent drying of media and cover plates in aluminum foil to prevent photo-reactivation and repair of UV-induced mutations. Incubate plates upside down at 30 °C for 2-4 days to allow for growth of viable yeast colonies (**Figure 1**).
7. Count the number of colonies, optionally with the help of a pen to mark off counted colonies, a hand held electronic counter pen, or a counter stand with magnification. Plot as a percentage of total plated cells at each μJ dose of UV irradiation to generate a survival curve for irradiated yeast (**Figure 2**).

NOTE: Haploid yeast strains can be expected to require lower doses of UV irradiation relative to diploid strains to reach the same percent survival. A strain of yeast lacking functional DNA repair enzymes, such as the *rad1* *S. cerevisiae* mutant, can be used as a positive control to indicate the presence of UV irradiation at very low doses.

8. Determine the dose of UV mutagenesis to be used for screening by referring to the survival curve established in 1.1.7. The x value of the point along the survival curve where y equals 50 is the UV irradiation dose at which 50% of yeast survive. Screening mutants at this low percent survival may result in a higher yield of successfully mutated strains, particularly for diploid yeast. The 50% survival dose for WT *S. boulardii*, as shown in **Figure 2**, is approximately 18,000 μJ .

NOTE: Although such high UV doses increase the risk of mutations in genes for cellular pathways other than the auxotrophic marker gene of interest, this drawback must be balanced against the need to induce mutations in both copies of the auxotrophic marker gene. For haploid strains in which only one gene copy must be mutated, screening at a higher percent survival, such as at 90%, decreases the risk of additional mutations and still allows for sufficient generation of auxotrophic mutants.

2. UV mutagenesis and screening for auxotrophic yeast strains

1. Prepare yeast as described in steps 1.1.1-1.1.3.
2. Expose yeast to the dose of UV irradiation corresponding to 50% survival, as determined in 1.1.8. For WT *S. boulardii*, this dose was determined to be approximately 18,000 μJ (**Figure 2**).
3. Collect 1 ml volumes of UV irradiated yeast and pellet by centrifugation in a microcentrifuge at 16,000 x g for 1 min. Aspirate supernatant and resuspend cells in 100 μl sterile water.
 1. Selection of mutants
 1. If using selection such as with *ura3⁻* auxotrophic mutants, plate irradiated yeast onto media containing 5-fluoroorotic acid (5-FOA) to select for cells lacking a functional Ura3 enzyme.
NOTE: Any yeast containing functional copies of Ura3 will convert 5-FOA to the toxin 5-fluorouracil, leading to cell death and allowing for easy selection of *ura3⁻* colonies that lack a functional Ura3²⁷. Analogous selection approaches are possible for the *LYS2* and *LYS5*; *TRP1*; and *MET2* and *MET15* markers using media containing α -aminoadipic acid²⁸; 5-fluoroanthranilic acid²⁹; and methyl mercury^{30,31}, respectively.
 2. Pipette the 100 μl of resuspended cells onto minimal media containing 5-FOA and use a sterile spreader to evenly coat the plate. Wrap plates in Parafilm and incubate upside down at 30 °C for 2-4 days to allow for growth of viable yeast colonies.
 3. Confirm the *ura3⁻* phenotype of any colony appearing on 5-FOA plates by restreaking onto YPD, uracil⁻ and 5-FOA plates (**Figure 3**). Use the tip of a sterile toothpick to collect part of a single colony and gently drag the cells across fresh YPD, uracil⁻, and 5-FOA plates. Again incubate wrapped plates upside down at 30 °C for 2-4 days.
NOTE: Viable colonies will appear as raised, roughly circular growths while non viable cells will appear only as an opaque smear without any raised growths (**Figure 3**).
 2. Screening of mutants
 1. If generating an auxotrophic mutant for which selection methods are not available, prepare serial 1:10 dilutions of UV irradiated yeast cells in sterile water and pipette the dilutions onto YPD media, using a sterile spreader to evenly coat the plate.
 2. Wrap plates in Parafilm and incubate upside down at 30 °C for 2-4 days. Determine which dilution allowed for growth of individual colonies that can easily be distinguished from each other, usually no more than approximately 100 colonies per plate.
 3. Repeat UV mutagenesis of yeast samples as described in 1.2.1-1.2.3 and plate cells at this determined dilution. Pipette the diluted yeast onto YPD media, use a sterile spreader to distribute the cells, and incubate wrapped plates upside down at 30 °C for 2-4 days.
 4. Screen for auxotrophs by replica plating onto selective media lacking the metabolite of interest. First, secure a sterile velvet pad onto a plate stand and invert the plate with UV irradiated colonies onto the velvet, marking the plate orientation.
 5. Next, invert a fresh plate lacking the metabolite of interest onto the velvet and lightly press down to transfer cells from the velvet to the plate. Store the original plate at 4 °C. Wrap and incubate the new plate upside down at 30 °C for 2-4 days.
 6. As an alternative to replica plating, screen mutants by re-streaking colonies from YPD onto selective media. Use the tip of a sterile toothpick to collect part of a single colony and gently drag the cells across a fresh YPD plate and a plate lacking the metabolite of interest. Again incubate wrapped plates upside down at 30 °C for 2-4 days.
NOTE: Care must be taken to select single colonies and streak out colonies multiple times to confirm a homogeneous population of true auxotrophic cells.
4. Further confirm the phenotype of irradiated cells by inoculating single colonies into 5-10 ml of both YPD and media lacking the appropriate metabolite (e.g., in uracil⁻ media for a *ura3⁻* mutant). Incubate on a roller drum at 30 °C O/N to confirm growth of cells in YPD media but not in the absence of the metabolite.
NOTE: Although growth patterns on solid media should clearly indicate yeast auxotrophic status, it is possible for some yeast to tolerate stresses and form small, slow growing colonies on solid media but yet not tolerate the same conditions in liquid media (unpublished observations). Inoculation into liquid cultures should thus be performed to thoroughly confirm growth patterns of UV irradiated mutants.
5. For long term storage of confirmed auxotrophic mutants, prepare glycerol stocks by inoculating cells into 10 ml YPD and incubating on a roller drum O/N at 30 °C. Pellet cells by centrifugation for 3 min at 2,500 x g and aspirate media. Resuspend cells in 50% sterile filtered glycerol, transfer to a cryovial, and store at -80 °C.

NOTE: UV mutagenized yeast potentially contain mutations in multiple genes other than in the auxotrophic marker of interest. Before continuing with use of verified auxotrophic mutants, these strains should be further analyzed through gene sequencing and assessment of resistance to pH, bile acid stresses, and other characteristics relevant to probiotic strains, as described elsewhere². Additionally, use of PCR homology or CRISPR/Cas9 targeting to more selectively mutate auxotrophic markers should be considered as an alternative to UV mutagenesis⁴⁻⁶.

2. Yeast Transformation

1. LiOAc Transformation of Yeast

1. Inoculate single yeast colonies into 5-10 ml of YPD media and incubate on a roller drum at 30 °C O/N.
2. To induce log phase growth and increase efficiency of plasmid uptake, determine cell concentration using a spectrophotometer to measure a 1:10 dilution of cells in sterile water in a plastic cuvette. Dilute O/N cultures to an OD₆₀₀ of 0.16-0.2 (approximately 2×10^6 - 2.5×10^6 cells/ml) in 50 ml of fresh warm YPD and incubate cells on an orbital platform shaker set to 200 rpm until the culture reaches approximately 1×10^7 cells/ml, usually around 4 hr.

NOTE: Transformation efficiency can be measured as a function of the number of successfully transformed yeast colony forming units (CFU) per µg of plasmid DNA. Increased efficiency results in more transformed colonies per µg of plasmid DNA. Subculturing yeast cells and collection during log phase growth is one factor that increases transformation efficiency¹².

3. Pellet cells by centrifugation at 2,500 x g for 3 min.
4. Aspirate the supernatant and transfer cells to a 1.5 ml microcentrifuge tube by resuspending the pellet in 1 ml sterile water.
5. Pellet cells by centrifugation at 16,000 x g for 1 min in a microcentrifuge, aspirate the supernatant and wash cells by resuspension in 1 ml TE/LiOAc (Tris EDTA LiOAc) buffer.
6. Repeat centrifugation and resuspend cells in TE/LiOAc buffer to a concentration of 2×10^9 cells/ml.
7. Prepare transformation mixtures with each of the following: 50 µl of prepared yeast in TE/LiOAc buffer, 5 µl of carrier DNA (10 µg/µl), and 1 µl of plasmid DNA (1 µg). Micrograms of plasmid DNA may be titrated as increasing amounts of DNA may or may not lead to increased transformation efficiency³².

NOTE: For a mutant yeast strain lacking one auxotrophic marker, only one plasmid encoding the marker can be transformed per sample. Furthermore, use of a plasmid encoding an easily detectable protein, such as GFP, will allow for efficient determination of proper folding and expression of heterologous protein by the yeast strain subsequent to transformation.

8. To each preparation, add 300 µl of PEG/LiOAc/TE and vortex thoroughly. Incubation of intact cells with PEG is essential for efficient transformation¹³.
9. Incubate preparations at 30 °C for 30 min with agitation by placing microcentrifuge tubes in a beaker placed onto an orbital platform shaker at 200 rpm.
10. Add 35 µl of DMSO to each reaction and heat shock cells for 15 min in a 42 °C water bath. Although there are conflicting reports of the added benefit of DMSO³³, heat shock of intact yeast cells has been shown to greatly increase transformation efficiency¹².
11. Wash cells by pelleting via centrifugation as in 2.1.5, aspirating or pipetting off the supernatant, and resuspending in 1 ml sterile water. Gently pipette up and down to break up the cell pellet.

NOTE: It is critical to thoroughly remove the supernatant because the media used in generating competent cells can inhibit yeast growth and colony formation.

12. Repeat cell pelleting as in 2.1.5, aspirate the supernatant, and resuspend the cells in 100 µl sterile water. Pipette the full volume onto a selective plate and use a sterile spreader to evenly coat the plate with transformed yeast.
13. Wrap edges of coated plates in Parafilm to prevent drying of media and incubate upside down at 30 °C for 2 days to allow for growth of transformed yeast cells. Successful, efficient transformation and auxotrophic selection of *Saccharomyces cerevisiae* yields a high number of colonies per transformation preparation, although yield can be much lower for other strains (**Figure 4**).
14. Store yeast plates for the short term (generally 1-3 weeks) upside down and covered at 4 °C. Prepare glycerol stocks of transformed yeast as described in 1.2.5 for long term storage.

NOTE: Further studies testing transformed CFU are necessary to determine plasmid stability and evaluate proper expression of heterologous protein. Thorough descriptions of plasmid stability³⁴, use of immunoblotting to detect denatured proteins recovered from cell samples¹⁷, enzyme linked immunosorbent assay (ELISA) to detect properly folded three dimensional proteins¹⁶, and the use of GFP in yeast studies³⁵ are available elsewhere. **Figure 6** shows a representative image of successful GFP expression in transformed *S. cerevisiae* relative to untransformed cells. Use of such a fluorescent protein is one means of efficiently determining successful heterologous protein production.

2. Electroporation of Yeast

1. Inoculate single yeast colonies into 5-10 ml of YPD media and incubate on a roller drum at 30 °C O/N.
2. Determine cell concentration using a spectrophotometer to measure a 1:10 dilution of cells in sterile water. Dilute O/N cultures in 100 ml fresh warm YPD media to an OD₆₀₀ equivalent of approximately 0.3. Incubate subcultures at 30 °C on an orbital platform shaker set to 200 rpm until reaching an OD₆₀₀ of approximately 1.6, usually 4-5 hr. Each 100 ml subculture will generate enough conditioned cells for two transformation reactions.
3. Pellet cells by centrifugation at 2,500 x g for 3 min. Aspirate the supernatant and wash cells by resuspending in 50 ml ice cold sterile water. Repeat the wash by pelleting cells, aspirating supernatant, and resuspending in fresh 50 ml ice cold sterile water.
4. Pellet the cells again and resuspend in 50 ml ice cold electroporation buffer (1 M Sorbitol, 1 mM CaCl₂).
5. Repeat spin as in 2.2.3, aspirate supernatant, and resuspend cells in 20 ml 0.1 M LiOAc/10 mM DTT. Incubate cell suspension on a roller drum at 30 °C for 30 min. Preincubation of cells in LiOAc and DTT synergistically increases the efficiency of electroporation³⁶.
6. Pellet the cells as in 2.2.3, remove supernatant, and wash by resuspending in 50 ml ice cold electroporation buffer. Repeat centrifugation and resuspend cells in ice cold electroporation buffer to a final volume of 1 ml.
7. Prepare on ice: conditioned yeast cells, sterile electroporation cuvettes, and plasmid DNA. Immediately after final resuspension of conditioned cells in 1 ml electroporation buffer, combine 400 µl conditioned yeast cells with approximately 1 µg of plasmid DNA and

add to an ice cold 0.2 µm electroporation cuvette. Use of increased amounts of DNA may slightly increase transformation efficiency¹¹. Incubate reaction on ice for 5 min, then electroporate with electroporator set to 2.5 kV and 25 µF.

NOTE: As described in 2.1.7, a mutant yeast strain lacking one auxotrophic marker can be transformed with only one plasmid encoding the mutated marker per sample. Also, using a plasmid that encodes an easily detectable protein such as GFP allows for efficient determination of proper folding and expression of heterologous protein subsequent to transformation.

8. Transfer electroporated cells into 8 ml of a 1:1 mixture of YPD:1 M sorbitol and allow cells to incubate on a roller drum at 30 °C for 60 min.
9. Pellet cells as in 2.2.3 and resuspend in 100 µl 1:1 YPD:1 M sorbitol. Plate the full volume onto selective media containing 1 M sorbitol, wrap edges of plates in Parafilm, and incubate plates upside down at 30 °C for 2-5 days to allow for growth of transformed yeast cells.

NOTE: It is critical to test transformed yeast to evaluate proper expression of heterologous protein, as described in 2.1.14 and 2.2.7.

3. Oral Gavage of Mice with Transformed Yeast

1. Perform all animal care and handling procedures according to the Guide for the Care and Use of Laboratory Animals and Institutional Animal Care and Use Committee approval.
2. Prepare O/N yeast cultures by inoculating single colonies of transformed auxotrophic yeast into 5-10 ml of selective media. Incubate cultures O/N for at least 8 hr on a roller drum at 30 °C until saturated.
NOTE: Use of plasmid encoding test proteins such as GFP will allow for ease of protein expression testing in gavaged yeast, as described in 4.7.
3. For maximal induction of protein expression and to induce log phase growth, prepare subcultures from the O/N cultures by diluting to an OD₆₀₀ equivalent of approximately 0.16-0.2 in 50 ml of appropriate media as described in 2.1.2.
4. Determine the concentration of subcultured cells as in 1.1.2 and adjust to 10⁹ cells/ml. Prepare a 100 µl dose for each mouse, with a few hundred µl extra volume per group to improve accuracy and ease of sample loading.
5. Pellet cells by centrifugation at 2,500 x g for 3 min or in a microcentrifuge at 16,000 x g for 1 min. Aspirate supernatant and resuspend cells by adding an equal volume of sterile water and gently pipetting up and down.
6. Fix an appropriate gauge gavage needle (22 G for 15-20 g mice) onto a 1 ml sterile syringe and load yeast sample, being sure to eliminate any bubbles and set plunger to a 100 µl increment. Load an additional syringe with sterile water to gavage control mice and check for presence of any contaminating yeast.
7. Pick up the mouse to be gavaged using the non-dominant hand, with index finger and thumb tightly grasping the skin around the neck (**Figure 6A**). Tuck the tail under the small finger to prevent movement of the lower body. Be sure that the grip is secure and prohibits the mouse from moving its head in order to prevent damage to internal tissues during gavage.
NOTE: Estimate how far the gavage needle should be inserted by holding the needle against the mouse such that the bulb is even with the xiphoid process of the sternum. Inserting this length of the needle will typically allow the gavage needle bulb to enter the stomach.
8. Using the dominant hand, gently insert the gavage needle into the mouse esophagus by angling the needle along the roof of the mouth and back of the throat, keeping slightly to the left of center. Wait for the mouse to swallow the bulb of the needle and allow the needle to descend slightly further to the point estimated in 3.7 (**Figure 6B**). If any resistance is felt during insertion of the gavage needle or if the mouse at any time begins to gasp, gently remove the needle and again try to find the esophagus.
9. After the mouse has swallowed the bulb of the gavage needle, gently depress the syringe plunger to administer 100 µl (10⁸ CFU) of yeast directly into the mouse stomach.
NOTE: Although mice are unable to vomit any of the gavaged solution after administration^{18,19}, it is possible for reflux to occur during gavage^{21,37}. Proper insertion of the gavage needle, as well as adjusting the volume and viscosity of the solution, can help to limit reflux and ensure accurate dosing.
10. Carefully remove the gavage needle from the mouse stomach and esophagus and return the mouse to the cage. Check that the mouse is breathing and moving normally after gavage to ensure that the gavage needle was properly inserted throughout the procedure and that no solution was aspirated.

4. Harvest of Murine Peyer's Patches and Isolation of Viable Yeast Colonies

1. At the appropriate time point post gavage, typically 4 hr, sacrifice mice using IACUC approved methods. Check for lack of responsiveness following a toe pinch, and use a secondary measure such as cervical dislocation to sacrifice the mouse. Additional time points may also be tested, as numerous studies have shown that efficiency and timing of uptake across the epithelium is particle dependent^{38,39}.
2. Lay the mouse with the abdomen fully exposed and sterilize the abdominal area by spraying with 70% EtOH. Make a transverse incision through the fur and skin with scissors, being careful not to damage any internal tissues. Manually pry the incision open further to expose the peritoneum, the thin serosal lining covering the abdominal organs. Gently lift the peritoneum and make a transverse incision to expose the intestines.
3. Carefully use blunt forceps to tease the small intestine away from the mesenteric arteries, fat, and other tissues. Expose the small intestine from the stomach, in the upper left quadrant of the mouse abdomen, to the cecum, the large pocket of intestinal tissue at the start of the large intestine.
4. Isolate the Peyer's patches by looking for 1-3 mm roughly circular patches of opaque tissue along the small intestine (**Figure 7**). Using curved dissection scissors, cut away the dome of the Peyer's patch, leaving margins to ensure that none of the surrounding lamina propria is collected.
NOTE: Most mice have between 4-8 easily visible Peyer's patches. Performing the procedure in an area with direct overhead lighting will increase the ease with which Peyer's patches can be visualized.
5. Collect dissected Peyer's patches in complete Iscove's modified dulbecco's media (IMDM).
NOTE: It is critical to use sterile technique and include antibiotics in the collection media in order to prevent gastrointestinal bacterial contamination of yeast plates.

6. Strain Peyer's patches through a 40 μm cell strainer to eliminate collection media. Wash Peyer's patches with fresh complete IMDM over a 50 ml tube and use a plunger from a 1 ml syringe to gently break up the Peyer's patches. Pellet strained cells by centrifugation at 1,800 rpm for 7 min. Aspirate supernatant and resuspend cells in a final volume of approximately 100 μl .
7. Apply strained cells onto selective yeast media and use a plate spreader to evenly distribute cells. Wrap plate edges in Parafilm and incubate plates upside down at 30 °C for 2 days to allow for growth of any viable yeast recovered from the murine Peyer's patches.
NOTE: Further studies after recovery of yeast from Peyer's patches are necessary to confirm that the strains are able to deliver properly folded heterologous protein to these immune tissues. As described in 2.1.14, such methods may include immunoblotting, ELISA, or fluorescence microscopy to detect fluorescent proteins such as GFP^{2,40}.

Representative Results

Generation of a survival curve following UV irradiation requires plating of diluted yeast cells such that distinct colony forming units (CFU) are able to form. Each 500 μl sample collected as described above contains approximately 5×10^6 cells; however, greater than 100 colonies per plate are difficult to accurately distinguish. Plating undiluted sample as well as serial 1:10 dilutions of irradiated cells thus ensures that CFU can be enumerated at each UV dose, as demonstrated in **Figure 1**. The CFU count, multiplied by the dilution factor, is then divided by the total number of original irradiated cells in each 500 μl sample in order to determine percent survival at each dose. **Figure 2** shows the calculated percentage of diploid wild type *S. boulardii* cells able to survive 0 μJ , 5,000 μJ , 10,000 μJ , 15,000 μJ , 20,000 μJ , 22,500 μJ , 25,000 μJ , 35,000 μJ , and 50,000 μJ . These data establish a clear curve that can be used to find the dose corresponding to 50% survival.

After selection of UV dose and irradiation of yeast cells, it is critical to screen mutant colonies to confirm lack of a functional auxotrophic marker gene. Use of a selection method, as described in 1.2.3.1 and shown in **Figure 3**, significantly increases the efficiency of phenotype confirmation. Shown is an example of *URA3* selection that takes advantage of the conversion of 5-FOA to the toxin 5-FU by intact *Ura3*. Analogous approaches are available for *LYS2* and *LYS5*; *TRP1*; and *MET2* and *MET15* and increase efficiency of selection for these mutations. Care must be taken to select individual colonies during screening. The consistent growth of mutant colonies on YPD and 5-FOA, but not uracil, plates indicates auxotrophic phenotype.

Figure 4 shows transformation efficiency for wild type *S. boulardii* (*S.b.*) relative to a commonly used laboratory *S. cerevisiae* strain (*S.c.*) using both the LiOAc (LiOAc) and electroporation (Electro) techniques. Although LiOAc transformation is very efficient for *S. cerevisiae*, transformation efficiency for *S. boulardii* is greatly improved using electroporation. **Figure 5** shows use of fluorescence microscopy as an example method of analyzing proper protein expression from transformed yeast. Brightfield (A) and fluorescence (B) images are shown for *S. cerevisiae* transformed with a *URA3* plasmid encoding GFP, demonstrating functional expression of heterologous protein from the transformed yeast. Cells can be immobilized for better visualization using coverslips coated in concanavalin A (coat 5 μl of a 2 mg/ml stock solution in water onto each 22 x 22 μm coverslip and air dry).

Figure 6A shows a C57BL/6 mouse held just prior to oral gavage. The hand grasps the back and neck of the mouse firmly such that the mouse is not able to move the head in any direction. This hold allows the gavage needle to be placed accurately and with decreased risk of tissue damage. **Figure 6B** shows the gavage needle held after the mouse swallows the gavage needle. Following the incubation period, the small intestine of the sacrificed mouse should be carefully teased apart from the surrounding tissues, as shown in **Figure 7**. This manipulation allows for easy identification of Peyer's patches and for clean dissection of the patches without collecting any of the surrounding lamina propria. Finally, **Figure 8** shows typical recovery of viable yeast CFU from Peyer's patches.

Solutions	Yeast Media and Plates	Transformation Reagents
Polyethylene glycol (PEG) 50%:	YPD:	TE/LiOAc:
250 g PEG 3350	20 g peptone	50 ml 10x TE
500 ml sterile water	20 g dextrose	50 ml 10x (1M) LiOAc
Filter sterilize	10 g yeast extract	400 ml sterile water
	1 L water	Filter sterilize
	Autoclave	
TE 10x:	YPD plates:	PEG/TE/LiOAc:
100 mM Tris	20 g peptone	400 ml 50% PEG
10 mM EDTA	20 g dextrose	50 ml 10x TE
pH to 7.5 and filter sterilize	20 g agar	50 ml 10x (1M) LiOAc
	10 g yeast extract	
	1 L water	
	Autoclave	
20% glucose:	Uracil⁻ selective media	Carrier DNA (SS DNA):
200 g dextrose	2 g amino acid mix lacking uracil	Store at -20 °C and prior to use heat for 1-2 min at 100 °C to melt strands and store on ice
1 L water	6.7 g yeast nitrogen base without amino acids	
Filter sterilize	1 L water	
	Sterilize by autoclaving or sterile filtering	
	Add 20% glucose 1:10 before use	
50% glycerol:	Uracil⁻ plates:	Electroporation buffer:
500 ml glycerol	In a 250 ml flask:	1 M Sorbitol
500 ml water	2 g amino acid mix lacking uracil	1 mM CaCl ₂
Autoclave	6.7 g yeast nitrogen base without amino acids	Fill with distilled water
	150 ml water	Autoclave and store at 4 °C
	In a 2 L flask:	
	20 g agar	
	750 ml water	
	Autoclave flasks separately, then mix together with 100 ml 20% glucose	
Complete IMDM	5-FOA⁺ plates:	LiOAc/DTT
500 ml Iscove's Modified Dulbecco's Media	Autoclave in a 2 L flask:	0.1 M LiOAc
5 ml penicillin streptomycin glutamine 100x	20 g agar	10 mM DTT
500 µl 2-mercaptoethanol	750 ml water	
10% heat inactivated fetal bovine serum	Mix:	
2.5 ml sodium pyruvate 100 mM	6.7 g yeast nitrogen base without amino acids	
	2 g amino acid mix without uracil	
	150 ml warm water	
	When cool, add:	
	0.05 g uracil powder	
	1 g 5-FOA	
	Stir and filter sterilize	
	Add to autoclaved agar solution	
	Mix with 100 ml 20% glucose	

Table 1. Reagents List. Described are the reagents needed for making each of the solutions, yeast media and plates, and transformation buffers used for the protocols in this manuscript.

Figure 1. Yeast colonies grown on YPD media. Example YPD plate showing viable colony forming units (CFU) of probiotic yeast after UV irradiation. Cells were serially diluted such that individual CFU can be distinguished and counted. [Please click here to view a larger version of this figure.](#)

Figure 2. Survival curve for diploid probiotic yeast. Number of viable *S. boulardii* CFU as a percent of total plated cells was plotted for each μJ dose of UV irradiation (solid line). The vertical red line indicates the μJ UV dose corresponding to 50% survival of this yeast strain. A *rad1 S. cerevisiae* mutant, which cannot repair damage from UV mutagenesis, is shown as a control (dashed line). [Please click here to view a larger version of this figure.](#)

Figure 3. Confirmation of *ura3⁻* phenotype of UV irradiated cells on YPD, uracil, and 5-FOA plates. Cells from individual UV mutant colonies were collected using the tip of a sterile toothpick and gently streaked across YPD, uracil, and 5-FOA plates. Cells were first streaked in two perpendicular crossing lines, then a new toothpick was used to pass through the second line and continue spreading cells until individual cells separate. A true *ura3⁻* mutant (*mut*) grows on YPD media and in the presence of 5-FOA, but not in the absence of uracil. Control *ura3⁻* *S. cerevisiae* (*ura3⁻*) and *URA3⁺* *S. boulardii* (*URA3⁺*) are shown for comparison and to confirm proper preparation of yeast media. [Please click here to view a larger version of this figure.](#)

Figure 4. Transformation Efficiency of *Saccharomyces* strains. Wild type *S. boulardii* (*S. b.*) and a laboratory *S. cerevisiae* strain (*S. c.*) were transformed using the described LiOAc (LiOAc) and electroporation (Electro) protocols. Results are plotted as mean CFU obtained per µg of plasmid encoding a kanamycin resistance marker. Bars show the mean of duplicate experiments with error bars depicting the standard error of the mean. [Please click here to view a larger version of this figure.](#)

Figure 5. Functional Protein Expression by Transformed Yeast. *S. cerevisiae* transformed with empty plasmid (A) and plasmid encoding GFP (B) were analyzed using a fluorescent microscope. Fluorescence in the yeast cells transformed with GFP plasmid indicates successful production of functional GFP. [Please click here to view a larger version of this figure.](#)

Figure 6. Proper handling of a C57BL/6 mouse for oral gavage. The mouse is held tightly in the non-dominant hand with the tail tucked under the small finger so that no movement is possible (A). The gavage needle is inserted into the pharynx along the roof of the mouth. The mouse is allowed to swallow the bulb of the gavage needle, allowing the solution to then enter the stomach as the plunger is depressed (B). [Please click here to view a larger version of this figure.](#)

Figure 7. Preparation and dissection of Peyer's patches. The small intestine is shown dissected away from the other internal organs and tissue, with arrows pointing to a few of the Peyer's patches. [Please click here to view a larger version of this figure.](#)

Figure 8. Yeast Recovery from Peyer's Patches. An example of viable CFU detected after dissection, homogenization, and plating of total Peyer's patch cells from a mouse gavaged with *S. boulardii*. Cells were plated onto YPD yeast media and incubated at 30 °C for 2 days. Typical yield of CFU recovered per mouse is less than 10. [Please click here to view a larger version of this figure.](#)

Discussion

Together, the protocols herein describe the essential steps necessary for the development and testing of auxotrophic probiotic yeast strains for delivery of heterologous therapeutic protein to the intestine. This manipulation and testing of recombinant probiotic yeast requires techniques and resources with which any individual laboratory may not currently be familiar. Thus, although numerous previous studies have described the above protocols for multiple yeast and mouse strains, these methods have not to the authors' knowledge been presented in a detailed, unified

form. Furthermore, the present manuscript places particular emphasis on adapting current standardized protocols for the genetic manipulation of probiotic yeast, which are less well characterized than commonly used laboratory yeast strains. Many steps for both mutagenesis (discussed in part 1) and transformation (part 2) must be optimized for the manipulation of such diploid, probiotic yeast isolates. This manuscript also discusses potential pitfalls associated with animal handling (part 3) and dissection of the Peyer's patch immune tissues of the small intestine (part 4).

As many industrial and clinically relevant yeast strains are not immediately adaptable to large-scale genetic manipulation, it is first necessary to generate strains such as auxotrophic mutants that can be grown and selected without expensive antibiotics. UV mutagenesis is one such approach that allows for quick nonspecific mutation of auxotrophic genes^{7,41}. Survival curves can easily be generated (**Figures 1 and 2**) to determine the appropriate dose for screening mutants. However, this approach carries the risk of inducing off target mutations that may affect growth rate or other properties of the yeast strain. Targeted knockouts can instead be generated using PCR constructs or the CRISPR/Cas9 system. Subsequent screening or selection (**Figure 3**) of mutants allows for identification of auxotrophic yeast. Use of selection by plating onto 5-FOA media, for example, allows for rapid elimination of any yeast still containing a functional *URA3* auxotrophic gene. When possible, this selection approach may be preferable to a screen, which requires analysis of all colonies generated. With either selection or screening, however, it is critical to perform repeated streaking of individual yeast colonies onto selective media to confirm auxotrophic status.

Transformation of the generated mutants can be accomplished through different protocols. Although LiOAc transformation is effective in the transformation of many yeast strains, particularly for the most commonly used laboratory *S. cerevisiae* strains, alternative protocols such as electroporation may transform other yeast isolates with greater efficiency (**Figure 4**). Each new strain should be tested using multiple protocols to determine the optimal conditions for transformation. Varying incubation times and concentration of DNA, for example, can influence overall transformation efficiency and should be tested and optimized for each strain³³.

Oral gavage allows for the delivery of controlled doses of these recombinant yeast directly to the murine gastrointestinal tract, whose immune tissues can then be assayed for yeast and heterologous protein. Proper oral gavage technique (**Figure 6**) is critical to minimize animal discomfort and increase experimental precision. Furthermore, the Peyer's patches are key sites to assess uptake of recombinant yeast from the intestine. These clusters of immune tissue are important sites of antigen sampling and induction of mucosal immune responses. Large antigens, including yeast 3–6 µm in diameter, are most likely to be taken up by the M cells of Peyer's patches in order to cross the gastrointestinal epithelium and interact with immune cells. Care must be taken when dissecting the Peyer's patches to ensure that only cells from within the patch rather than the intestinal lumen or lamina propria are collected (**Figure 7**). Further steps must also be taken following dissection to assess proper expression and function of heterologous protein in the recovered yeast (**Figure 8**). Preparation of total protein from yeast lysates and immunoblotting is one standard method to assess protein expression; however, this approach does not provide information regarding protein folding and function. To assess protein function, yeast can be transformed with a plasmid encoding GFP and analyzed under a fluorescent microscope after recovery from Peyer's patches to assess functional GFP expression (**Figure 5**).

In sum, this manuscript presents a unified set of detailed experimental protocols spanning steps from the generation of auxotrophic mutants to the recovery of probiotic yeast from the murine intestine. By compiling protocols that do not traditionally fall within a single area of expertise, these descriptions will facilitate further studies testing immunological responses to probiotic yeast designed as oral drug delivery vectors. The authors hope this study will encourage discussion and promote optimization of experimental methods for each yeast strain tested, paving the way for the most efficient approaches to the development of novel, probiotic-based recombinant therapies.

Disclosures

The authors have nothing to disclose.

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