

EXPERIMENTAL EVOLUTION IN YEAST: A PRACTICAL GUIDE

Maitreya J. Dunham

Contents

1. Introduction	488
2. Experiment Rationale	488
3. Experimental Evolution Approaches	490
3.1. Serial dilution	490
3.2. Chemostats	491
3.3. Turbidostats	492
3.4. More specialized systems	493
3.5. Miniaturization	493
4. Experimental Design	493
4.1. Growth conditions	493
4.2. Population size	494
4.3. Experiment duration	495
5. Practical Considerations	496
5.1. Strains and markers	496
5.2. Media	497
5.3. Growth rate	498
5.4. Good sterile practices	499
5.5. Good strain hygiene	499
5.6. Record-keeping	500
6. Analysis Techniques	500
6.1. Sampling regimen	500
6.2. Population genetics	501
6.3. Fitness	501
7. Example Protocol	502
7.1. Medium formulation	502
7.2. Chemostat preparation	502
7.3. Chemostat assembly	503
7.4. Inoculation	503
7.5. Daily sampling	503

Department of Genome Sciences, University of Washington, Seattle, Washington, USA

Methods in Enzymology, Volume 470
ISSN 0076-6879, DOI: 10.1016/S0076-6879(10)70019-7

© 2010 Elsevier Inc.
All rights reserved.

7.6. Weekly sampling	503
7.7. Analysis	504
8. Conclusions	504
Acknowledgments	505
References	505

Abstract

Experimental evolution refers to a broad range of studies in which selection pressures are applied to populations. In some applications, particular traits are desired, while in others the subject of study is the mechanisms of evolution or the different modes of behavior between systems. This chapter will explore the range of studies falling under the experimental evolution umbrella, and their relative merits for different types of applications. Practical aspects of experimental evolution will also be discussed, including commercial suppliers, analysis methods, and best laboratory practices.

1. INTRODUCTION

Experimental evolution is a generic term for laboratory selection experiments beyond those requiring simple one-step mutagenesis but perhaps more restricted in scale than the longer term pressures associated with domestication or geological timescale evolution. As our ability to analyze whole genome sequences improves via microarray and sequencing-based methods, we can expect more problems to become accessible through experimental selection approaches.

In this chapter, I will cover the different types of selections typically performed under the guise of experimental evolution, citing a limited selection of example cases, and then move to the practical considerations involved in undertaking a subset of these. The many scientific contributions of experimental evolution in viral, microbial, and animal systems will not be covered in this chapter. However, the reader will find many excellent reviews that cover these systems ([Adams, 2004](#); [Buckling *et al.*, 2009](#); [Burke and Rose, 2009](#); [Elena and Lenski, 2003](#); [Garland and Kelly, 2006](#); [Philippe *et al.*, 2007](#); [Zeyl, 2006](#)).

2. EXPERIMENT RATIONALE

Rationales for experimental evolution approaches in yeast are as numerous as the practitioners. Many research groups see the promise in explicitly testing many of the tenets of modern evolutionary theory. Experiments with sex and ploidy are exemplars of this approach, and have been

reviewed thoroughly elsewhere (Zeyl, 2004). Other evolutionary questions subjected to experimental testing include the role of mutation rates (e.g., Thompson *et al.*, 2006), mechanisms of assortative mating (Leu and Murray, 2006), cooperation (Shou *et al.*, 2007), and clonal interference (Kao and Sherlock, 2008).

In its simplest guise, such as selection experiments on drug resistance, laboratory populations can mimic the types of long-term adaptation that occur in chronic infections or cancer progression. Because the selection pressures can be carefully controlled under laboratory conditions, however, mutations can be more carefully assigned to a variable than by examining clinical samples. Acquisition of fluconazole resistance is a fine example from the *Candida* literature (e.g., Cowen *et al.*, 2000). These experiments serve as both a model for the development of drug resistance, and for the unraveling of the molecular mechanisms underlying resistance to particular drugs.

Other types of experiments essentially extend this concept of the mutant or suppressor screen. With longer term, less severe selection pressures than with viability-based selection schemes, more subtle mutations, including combinations of such mutations, may be recovered. Although the yeast genome deletion collection provides an interesting set of mutants for the assay of phenotypes, the strains represent only null alleles. To make progress in further dissecting genetic pathways, the field may benefit from a return to the rare but interesting alleles generated by spontaneous mutation, particularly for essential genes.

This style of experimentation can also shed light on larger questions of systems biology, such as evolution of gene expression, the relative merits of regulatory versus structural mutation, whether mutations affecting control points in a network are more wide-acting, and the mutability of different gene targets. Metabolic selection pressures have been particularly useful for studies along these lines (e.g., Ferea *et al.*, 1999; Francis and Hansche, 1972; Gresham *et al.*, 2008; Hansche *et al.*, 1978).

Interesting questions about genome structure and organization can also be answered using the results of experimental evolutions. For example, point mutations, transposon insertions, and copy number variants have all been recovered from selection experiments (e.g., Blanc and Adams, 2003; Brown *et al.*, 1998; Dunham *et al.*, 2002; Gresham *et al.*, 2008). The types of effects generated by these different classes of genetic alterations, and the relative accessibility of different gene targets to each type of lesion, are still unexplored but accessible by these techniques. For example, copy number changes may be the most effective route by which to change gene expression level, but the ability of a gene to change copy number may in turn be determined by the proximity to repetitive DNA segments that facilitate copy number change, and further complicated by the pleiotropic effects of additional neighboring genes on an amplicon. Point mutation, on the other

hand, represents a more surgical approach to perturbing gene function, but single point mutations may rarely provide large expression changes.

Finally, experimental evolution can provide a facile technique to optimize or even create desired traits, for example in bioproducts, food, and beverage production (reviewed in [Verstrepen *et al.*, 2006](#)). Industrial yeast geneticists have long used this strategy successfully, often with strains of unknown genotype. Although recombinant DNA techniques in food-related industries such as wine and beer production are becoming more widespread (reviewed in [Schuller and Casal, 2005](#)), there is consumer reluctance to use such products. In such cases selection is frequently the only acceptable tool for improving extant yeast strains.

For more industrial processes, introduction of recombinant DNA into a strain is less of a concern, but there is to date no clear recipe for the rational design of metabolic networks. Selection without prior knowledge of the mechanism can instead improve the performance of yeast strains involved in processes such as production of fuel ethanol and biotechnology products. Transfer of exogenous synthesis pathways into yeast, for example, could be followed by selection for more efficient integration into the yeast network, or higher product production.

Applications to synthetic biology (one such approach is reviewed in [Saito and Inoue, 2007](#)) and synthetic ecology (e.g., [Shou *et al.*, 2007](#)) will also put experimental evolution techniques in the forefront. Allowing the processes of mutation and selection to tune synthetic constructs may be the most efficient way both to create such circuits and to better understand what is required to achieve optimal performance. Clever selection schemes will no doubt be necessary to push these systems in the right direction.



3. EXPERIMENTAL EVOLUTION APPROACHES

Laboratory evolution experiments fall into two broad categories: serial batch transfer and continuous culture. The most suitable approach depends on technical, practical, and scientific considerations, covered in the following sections.

3.1. Serial dilution

Serial dilution generally refers to selection performed in the standard growth regimes typically used in the lab: flasks, test tubes, solid media, or 96-well plates. Cultures are usually allowed to grow through a normal growth curve, with daily transfer of a small volume of the expanded culture into fresh medium. Serial dilution has many advantages: the materials necessary are typically already present in the lab and require no special engineering.

Conditions can be adjusted as the experiment progresses (e.g., drug concentrations increased as drug resistance improves). Selection pressures of a number of types can be accommodated. The easiest selections to understand are improvements to growth when maximal performance is attenuated either by exogenous or genetic means. In these cases, full growth curves may not be desired, as improved performance with respect to nutrient exhaustion or stationary phase may be separate outcomes unrelated to the main selection applied by the experimenter.

Nutrient exhaustion is a popular selection scheme for batch transfer experiments, brought to prominence by experiments in bacteria by Lenski and colleagues (reviewed in [Elena and Lenski, 2003](#)) and adapted for yeast by [Zeyl \(2005\)](#). Here, one nutrient is lowered to the point that it uniquely runs out first and limits the saturated biomass of the culture. The relative amount of time cells spend in each phase of growth may change over the course of one of these experiments, particularly as lag phase shortens and maximal growth rate improves.

Plate-based selection allows even more control over the transfer step, with visual identification of colonies. Either obviously larger or otherwise morphologically desired (e.g., [Kuthan *et al.*, 2003](#)) candidates can be serially inoculated to fresh plates, or, on the other end of the spectrum, as little selection as possible can be imposed by selecting random colonies. The latter approach has been used to generate mutation accumulation lines ([Zeyl and DeVisser, 2001](#)).

3.2. Chemostats

Chemostats have long been another favored platform for experimental evolution (reviewed in [Dykhuisen and Hartl, 1983](#)), and were, in fact, invented for this application ([Monod, 1950](#); [Novick and Szilard, 1950a,b](#)). A chemostat is a growth vessel into which fresh medium is delivered at a constant rate and cells and spent medium overflow at that same rate. Thus, the culture is forced to divide to keep up with the dilution, and the system exists in a steady state where inputs match outputs. The chemostat is attractive due to the enormous amount of control that is possible: growth rate, cell density, and selection pressure are all independently set. Because of these advantages, chemostats are also being used as tools for studying aspects of cell biology such as ammonium toxicity ([Hess *et al.*, 2006](#)), growth rate control (e.g., [Brauer *et al.*, 2008](#)), and comparative gene expression for mutants that would otherwise be difficult to compare due to profound growth rate differences (e.g., [Hayes *et al.*, 2002](#); [Torres *et al.*, 2007](#)).

Unlike serial transfer, chemostats require more specialized equipment, which can range from rather inexpensive (<USD\$10,000) systems assembled from available parts to elaborate custom fermenters costing upward of USD\$100,000. A table of suppliers and plans is provided ([Table 19.1](#)).

Table 19.1 Fermenter suppliers

Maker	Web site or citation
<i>Commercial</i>	
ATR	http://www.atrbiotech.com/
Infors HT	http://www.infors-ht.com/
New Brunswick	http://www.nbsc.com/
Dasgip	http://www.dasgip.com/
Applikon	http://www.applikon-bio.com/
Sartorius	http://www.sartorius-stedim.com
<i>Homemade or custom-made</i>	
Tom Gibson and Ted Cox	Plans available in Gibson 1970 thesis, "The Fitness of an <i>E. coli</i> Mutator Gene," available through interlibrary loan from Princeton University
Reeves Glass (custom-made glassware)	http://www.reevesglass.com/
Gavin Sherlock	Plans available upon inquiry
G. Finkenbeiner (custom-made glassware)	http://finkenbeiner.com/

In choosing equipment, several experiment-specific questions are important to consider, including volume/population size, number of parallel experiments required, space constraints, and measurement and control needs. The simplest chemostat experiment requires a media-feed, volume-metering device, and growth vessel with overflow. pH control, dissolved oxygen monitoring, real-time data feeds, and other features, may be required for more complex experiments. Large volume (> 1 l) fermenters, such as those available from New Brunswick, Applikon, and ATR, offer in-line probes for such measurements. Small vessels can be made from modified laboratory glassware or with the assistance of a glass blower. Glass-blown designs may include a glass frit for aeration and a water jacket for temperature control in addition to sampling and media flow ports.

3.3. Turbidostats

Another continuous culture system, the turbidostat, first introduced by Bryson and Szybalski (1952), combines some properties of serial dilution and chemostats. Instead of adding new medium at a constant rate, in a turbidostat, cell density is held constant. This is achieved by a feedback loop allowing adjustment of the nutrient addition rate in response to changes in density, usually measured via light transmittance. Few commercial options appear to exist currently, but turbidostats can be built using modern

microprocessor controlled peristaltic pumps. Designs using simple light-measurement devices can be found in textbooks (e.g., [Norris and Ribbons, 1970](#)), and variations using LED and photodiode components would be straightforward extensions.

The turbidostat provides selection on maximal growth rate while simultaneously maintaining other conditions constant. The media composition defines the selection pressure as in other systems. Very little has been published on yeast grown in turbidostats, although that is likely to change given the benefits that this system provides.

3.4. More specialized systems

Other continuous culture systems have also been invented to control cell growth in various ways, via feedback at the level of pH or dissolved oxygen (generally known as auxostats, or, depending on implementation, accelerostats, see [Kasemets *et al.*, 2003](#)), dielectric permittivity (the permittostat, [Mark *et al.*, 1991](#)), or carbon dioxide (e.g., [Lane *et al.*, 1999](#)). Undoubtedly, many other variations are possible.

3.5. Miniaturization

Both serial dilution and chemostat culture can be greatly miniaturized. For example, microfluidic chemostats have been reported by [Groisman *et al.* \(2005\)](#). This can be a huge advantage when large numbers of replicates or single-cell resolution are required. Volume reduction can greatly affect the population size, though, which can in turn change how evolution proceeds (see the following section for further discussion). For now, microscale chemostats might best be used as a phenotyping tool to better characterize clones isolated from larger chemostat experiments.



4. EXPERIMENTAL DESIGN

There are a number of design considerations in planning any experimental evolution project. Several of the most important ones are covered in the following sections.

4.1. Growth conditions

The growth rate and selection pressures at which experimental evolutions are performed should be given careful thought. Selection upon maximal growth rate is preferred for many suppressor-type experiments, and may best be accomplished in serial dilution or turbidostat approaches because

chemostat cultures are difficult to operate near this value. To maintain a constant selection pressure, dilution should occur before the onset of growth limitation. In the event that entire growth curves are allowed each day (e.g., Zeyl, 2005), subpopulations with adaptations relevant to the different parts of the growth curve can be isolated. For example, strains with quicker resumption in lag, with faster maximal growth, or with additional ability to divide in stationary phase may all coexist in the culture. In such cases, the nutrient that runs out first is frequently termed limiting, but may be only one of several selection pressures.

In chemostat cultures, the selection pressure is mainly defined by the limiting nutrient. Limiting nutrient should always be explicitly tested in the chemostat conditions but can be prototyped in batch cultures by measuring the saturation density of cultures grown with varying amounts of the limiting nutrient. Confirmatory experiments should always be done in the exact conditions under which the real experiments will be performed. Complex selection pressures may not be perfectly modeled by batch experiments; for example, ammonium toxicity is only apparent under limiting potassium in the chemostat (Hess *et al.*, 2006). Micronutrients are another common culprit as hidden limitations (e.g., de Kock *et al.*, 2000). A comprehensive test of limiting nutrient would include demonstrating that density varies linearly with the nutrient of interest and not at all with other additives. The exact profile of limiting concentrations for various nutrients is strain-dependent and should be tested explicitly when working in different backgrounds.

The concentration of limiting nutrient is another key parameter since it determines cell density. Population size can greatly affect evolutionary parameters such as mutation supply, importance of drift, and time required for advantageous mutations to rise to detectable frequency (see further discussion in the following section). Also, density may affect gene expression to some degree. Given recent findings on quorum sensing in yeast cultures, more work remains to be done to understand density-dependent effects. There are also practical considerations such as sample volume required for accurate measurements: dry weight yield, for example, may require more material than an expression microarray using an amplification procedure. For continuous cultures in particular, sampling too much volume at once from the fermentor vessel can perturb the system, disrupting the steady state. When possible, passive sampling from the outflow is preferred, though this is not always possible, especially for time-sensitive applications such as expression measurements.

4.2. Population size

Beyond practical constraints, population size is a critical parameter for experimental evolution. In large populations, a modest adaptive mutation will take a long time to reach reasonable frequency in the population, and

clonal interference could be generating competitor clones simultaneously. In a small population, adaptation may be limited by the supply of beneficial mutations, and thus dominated by the highest frequency class. (See [Desai et al., 2007](#) for one treatment of these issues.) Per base mutation rate is on the order of 10^{-9} or 10^{-10} per site per generation, while per gene mutation rate to a null allele is closer to 10^{-6} . Given these estimates, populations of different sizes will sample vastly different subsets of the mutation landscape.

In serial dilution, two population size parameters must be determined: the saturation density and the bottleneck size. Severe bottlenecks may eliminate the vast majority of small-to-intermediate fitness variants that have not had time to reach appreciable frequency in a single day growth curve. In practice, the bottleneck population size is typically on the order of 10^6 – 10^7 cells.

Chemostat populations may be much larger, 10^{10} cells or more. Even in chemostat cultures, the initial phases may be dominated by variation generated as the culture grows from a single cell to the final population size. In this regime, the stochastic factor of when a mutation occurs can affect the allele frequency. For example, mutations of $\sim 10\%$ fitness advantage that become detectable starting around 100 generations of growth were hypothesized to fit this model ([Gresham et al., 2008](#)).

4.3. Experiment duration

The number of generations or length of time to allow a culture to evolve is both a practical and theoretical matter. In some cases, a particular desired outcome may be reached. In others, the end of an experiment may be governed by unfortunate circumstances such as contamination, user error, or infrastructure breakdown. Steps can be taken to prevent some of these events, such as careful attention to sterile practices. Backup power and aeration systems can also be implemented if necessary. Clumping of the culture is another less catastrophic, though perhaps less avoidable, endpoint (see below). Practical considerations concerning the number of manipulations and the necessity for lab worker attention past work hours may also limit experiment length.

Barring errors, however, experiments can run for days to decades. Whether experiments really require such long timescales is purely a scientific question. Early events in the chemostat may determine to a large extent what direction the population takes. Subsequent events may in fact be modifying mutations that optimize early events as opposed to “primary” events that may be more interesting. For example, where genome rearrangement is operative, amplicon size may shrink to contain relatively more causative genes and fewer copy number sensitive genes, or second-site suppressors of these sensitivities may arise.

Restarting interesting evolutions is also always an option, though there are likely added complications from loss of population complexity, plus added selection constraints on freeze tolerance and outgrowth from the stock. Evolved clones could alternatively be used as new founders.

5. PRACTICAL CONSIDERATIONS

5.1. Strains and markers

The strain used for experimental evolutions should be considered very carefully. Auxotrophies should be avoided for any metabolic selection, since selection would be strong for harnessing supplements as nutrient sources. Also, the network biology may be rather different in metabolically blocked strains. Marker genes can also cause fitness differences. [Baganz *et al.* \(1997\)](#) explicitly tested the fitness consequences of drug and nutritional markers in chemostat competition and found variation by selection pressure and marker used. In general, drug resistance markers were neutral while nutritional markers frequently caused fitness costs. These results are likely to depend strongly on the particulars of the growth regime and should be explicitly tested in novel environments.

Strain genotype beyond engineered genetic markers should also be considered. The classic S288C strain background, though a workhorse for decades of yeast genetics and biochemistry, has a number of probably lab-selected traits, including Ty insertions in *HAP1* and *CTR3*, increased petite frequency, and abnormal nitrogen source preferences. Almost all the lab strain alternatives (e.g., W303, sigma 1278b, and CEN.PK) share a large proportion of their genomes with this strain, though the exact alleles carried vary. These other backgrounds may also carry additional mutations, such as an adenylate cyclase (*CYR1*) mutation in CEN.PK. All lab strains are likely to contain some signatures of their domestication.

In addition, new mutations are generated spontaneously during lab cultivation and strain construction, and may result in hidden problems. One cautionary example can be found in a series of glucose-limited chemostat evolutions ([Ferea *et al.*, 1999](#)). During creation of the prototrophic ancestor strain, a loss of function mutation occurred in the gene *AEP3*, which stabilizes the RNA coding for subunits of ATP synthetase in the mitochondria. Not surprisingly, this mutation was detrimental in glucose-limited cultures, and reversion of this mutation was later found in evolved strains from two independent experiments ([Brauer *et al.*, 2006](#); [Dunham *et al.*, 2002](#); [Gresham *et al.*, 2006](#)).

Although such problems cannot be completely avoided, they can be mitigated by pairing compatibility of a particular strain with a particular selection pressure. For example, as long as a particular mutation is not

limiting, it may not be the target of beneficial mutation. [van Dijken *et al.* \(2000\)](#) undertook a comparison between strain backgrounds and found variation for all parameters tested, leaving no single strain with the “best” array of desired characteristics.

Flocculant growth is another strain feature that can present problems. In many experimental evolution regimes biofilm formation and clumping provide an advantage unrelated to the selection pressure of interest. For example, groups of cells may sink to the bottom of a fermentor, or biofilms may form on any surfaces, allowing subpopulations to avoid being diluted out of the culture. Besides complicating measurements of cell density, these subpopulations can contribute a constant supply of minority genotypes and interfere with population genetic measurements. Also, since many evolution experiments are designed around evaluating particular selection pressures, generic responses to the growth apparatus can be a confounding result.

For serial dilution-type experiments, some of this effect can be eliminated by transferring to a new vessel at each dilution rather than pouring off excess culture and adding new medium to the original vessel. In chemostats, transferring vessels is a riskier process, but can still be accomplished with care. Using strains with genotypes that limit their flocculation potential is another approach. Most lab strains carry at least one such mutation, and engineered FLO gene deletions (e.g., *flo8*) will not revert. Operation at high cell densities may further aggravate this phenotype, though much of this data is anecdotal. In practice, severe flocculation typically ends an experiment. Experiments can be prolonged by briefly sonicating culture samples before analysis.

Strains resulting from experimental evolution may also have a number of characters limiting their further use. Selection upon purely mitotic growth may relax selection on the rest of the yeast life cycle. Aneuploids, for example, may have trouble sporulating or segregate lethality resulting from the heterozygous deletion of essential genes. The mating pathway may also be abrogated as a means of conserving cellular resources ([Lang *et al.*, 2009](#)). Since most samples are archived through cryopreservation, freeze tolerance may be another hidden variable affecting later analysis. In addition, some cultures evolved in poor nutrient conditions may actually show growth deficiencies on rich media, though the reverse may also be true for the purposes of recovery from frozen stocks.

5.2. Media

Media requirements will depend on the desired selection pressure, but must be consistent no matter what the application. For this reason, rich media made from coarse or technical grade ingredients is not recommended due to batch variation. High-quality chemicals and water are required, especially

for nutrient-limited cultures, since trace contamination may provide a nontrivial amount of the nominal limiting nutrient. When possible, direct measurement of the limiting nutrient in media samples is recommended. Very sensitive spectrophotometric, enzyme-based assays are available commercially for many carbon sources. Phosphate can also be reliably measured by colorimetric assay. Other chemical analysis techniques such as inductively coupled plasma and mass spectrometry can more generally measure the elemental or metabolite profile of a sample, though sensitivity should generally be tested explicitly.

Preparation of media for experimental evolution requires more care than most microbiology experiments. Even small measurement errors can have profound effects on culture density. All materials used for media preparation should be rinsed thoroughly to prevent cross contamination. Variation in volume levels due to evaporation may be introduced by unanticipated differences in autoclave pressure, temperature, or timing and these may dramatically affect the outcome of the experiment. Filter sterilization of media into autoclaved carboys is one way of mitigating this effect. Some media components may be light or temperature labile. Also, in large volumes, viscous additives such as glucose may settle to create a gradient in the media vessel. Extra effort may be required to ensure that all such additives are thoroughly dissolved.

The growth apparatus itself may also leech chemicals into the medium. Metal fittings are one example supported by anecdotal evidence. Low-reactivity plastics and glass are typically a better choice for experiments that would be sensitive to such fluctuations. Plastics may pose their own problems if paired with incompatible solvents. Drug solutions requiring such reagents require particular care.

5.3. Growth rate

Growth rate is explicitly set by the experimenter for chemostat cultures, but the allowable range is dependent on media, temperature, and strain background, and growth rate differences introduce differences in many parameters. One important example is the ratio of respiration to fermentation in glucose-limited cultures grown at different rates. At growth rates below a strain-specific critical growth rate parameter, respiration dominates, but above this threshold, fermentation predominates. Evolutions performed very close to this boundary may shift thresholds as a mechanism of increasing efficiency. In chemostat and batch conditions, growth rate correlates strongly with a large gene expression pattern that overlaps that of the environmental stress response (Brauer *et al.*, 2008). Incorrect dilution settings can thus easily lead to spurious gene expression differences. A working rule of thumb is to keep settings within 10% of the target growth rate.

5.4. Good sterile practices

With some experiments running for years, contamination is a threat to experiment integrity. Contamination can be introduced during any break in continuity, most commonly during changing media supplies, or sampling from inside the vessel. Sampling should be done passively from the overflow if possible, though contaminants may also grow in exposed tubing. Periodic changes of this tubing may be required, particularly if drug-resistant contaminants interfere with detection of low-frequency variants from the main culture. This design also helps to prevent retrograde colonization of the main culture with contaminants from the effluent. Positive pressure provided by vigorous aeration is also recommended to limit contamination opportunities.

When changing media carboys, leakage should be avoided as much as possible. Droplets of media left around connectors provide a rich growth opportunity for microbes, and a risk of transfer to the inside of the tube during the next carboy transfer. Self-closing connectors are one preventative, though not entirely fail safe. Ethanol or bleach can remove most material, though again, such treatment is not fail safe.

Visual inspection of culture under the microscope or of colonies can detect high-frequency contaminants with morphological differences, such as bacteria or filamentous fungi. Experimental evolution frequently leads to morphological changes, so this is only appropriate for obviously different species. Also, some contaminants may not grow on solid medium. Checking that strain markers are constant over a time course can provide experimental assurance that other strains or species have not invaded. Strains with drug markers are more amenable to this test, but PCR-based markers can also be developed to differentiate between common strains. Obviously, if the contaminant is of the same strain background, problems will be more difficult to detect.

Another type of contamination is from the growth chamber into the media supply. Aerosolized yeast droplets can be pushed up the tubing if air pressure is forced through. Also, variants with improved flocculation capacity can lodge in crevasses at junction points. Clear tubing is recommended so that such colonization can be detected and problem spots eliminated. In extreme cases, the media input port may need to be heated to kill any back-contaminants. Wrappable heated tape is one common approach.

5.5. Good strain hygiene

Because mutations that arise during experimental evolution are generally not cloned using a functional assay, and because multiple mutations are typically present after sufficiently long timescales, strains should undergo limited passaging before introduction into the growth vessel to limit mutation accumulation. Even minor handling of strains can introduce lesions

(see *AEP3* example above). Preservation of a time zero sample of the population for comparison is important to eliminate these possibilities when performing *post hoc* analysis. Exact records of strain stock of origin for each population are also recommended, since ambiguities introduce uncertainty later in analysis.

5.6. Record-keeping

Because experimental evolutions may run for long periods of time, and be reanalyzed by many people within and between labs, good record-keeping practices are essential. An example system uses index cards for recording daily data, plus a digital copy of these records for analysis and archiving. A master database in Filemaker or some other software package can assist in keeping track of many experiments and their related data files, which should be backed up at regular intervals. Parameters such as strain background, media formulations, growth conditions, and other important details should be recorded. To identify potential problems with media composition, addition of new media supplies should be tracked.

Freezer stocks must be maintained in a very ordered way, particularly when lab personnel turn over. Systematic naming conventions are essential for long-term continuity. Freezer maintenance is also crucial to the long-term viability of evolved cultures. If possible, duplicates of glycerol stocks may be stored off site for backup purposes. Complex population samples are impossible to perfectly duplicate, so preplanning is required for this approach.

Sharing populations with other labs is another problem when unique samples are involved. Dense lawns or patches, or large numbers of isolated colonies, can be scraped from plates and frozen in glycerol culture to attempt to maintain population frequencies.

6. ANALYSIS TECHNIQUES

6.1. Sampling regimen

Obviously the details of what to measure day-to-day will depend heavily on what experiment is being performed. Parameters that may be recorded include cell density as surveyed by Klett colorimeter, spectrophotometer, or cell count; viable cell count on rich- or low-nutrient plates; notes on cell morphology, colony morphology, and flocculation status; and even changes in aroma. Frozen stocks may be collected daily. Small samples for processing into RNA and DNA may also be collected at intervals. If these samples are collected on a filter, a filtrate sample is generated simultaneously that can be assayed for residual nutrient or metabolite levels.

6.2. Population genetics

Population-scale expression and CGH measurements generated from these samples can be useful for interrogating the frequencies of copy number changes, though with the caveat that frequency changes and extra copy number changes in a subpopulation may look identical. Allele frequency measurements can also be made via quantitative PCR (Kao and Sherlock, 2008) or quantitative sequencing (Gresham *et al.*, 2008). Mutations of a variety of types can be measured via microarray (reviewed in Gresham *et al.*, 2008), though next generation sequencing is sure to contribute in the near future. With strong enough phenotypes, mutations can also be linkage-mapped using classical approaches or by bulk segregant analysis (Brauer *et al.*, 2006; Segre *et al.*, 2006).

Phenotype characterization methods can also vary. By definition, only phenotypes present in the selective conditions are relevant to the experiment at hand. However, particularly in the chemostat, it may be impossible to survey large numbers of samples for fitness or growth parameters. Bulk competition experiments provide one solution (e.g., Gresham *et al.*, 2008). If individual genotypes can be marked, or detected directly by sequencing, their frequency over time can be used to calculate their fitness. Assays on plates or nonselective media can also be used as a screen to narrow down the search space that needs to be covered in a more tedious growth state. However, plate phenotypes do not always behave as expected in the milieu of the population.

6.3. Fitness

Fitness is generally the most relevant phenotype in an experimental evolution, and can conveniently be assayed by direct competition experiments. Fitness measurements in particular should be optimally performed not just in the conditions under which the strain evolved, but even in the exact population context since fitness may be highly dependent on the competitors (Paquin and Adams, 1983a,b). This condition is almost impossible to recreate. In practice, fitness is generally assayed by direct competition with the ancestral strain or with other evolved clones. One or both strains may be marked to facilitate frequency measurements, or the relative frequency can be sampled by following mutations via quantitative sequencing or some other means.

Strain tagging with drug resistance markers is the most common way of performing mixed fitness assays (e.g., Gresham *et al.*, 2008; Paquin and Adams, 1983a,b). Fluorescence markers have also been used successfully (e.g., Kao and Sherlock, 2008; Thompson *et al.*, 2006), and are attractive because of both their ease of use and improved accuracy. While only hundreds to thousands of colonies can be easily assayed for drug resistance,

orders of magnitude more cells can be assayed by FACS. For both methods, accuracy improves as sampling density increases. One disadvantage of fluorescent markers is that expression of these proteins may impose a selective cost, which must be assayed in controls and subtracted out from all further measurements. Whether this cost is constant across conditions and strain backgrounds must be tested in each situation.

7. EXAMPLE PROTOCOL

Working from these general recommendations, this section describes an example glucose-limited chemostat evolution experiment. Related detailed protocols with photographs and recipes are available at <http://dunham.gs.washington.edu/>.

7.1. Medium formulation

Chemostat glucose-limited synthetic minimal media contains (per liter) 0.1 g calcium chloride, 0.1 g sodium chloride, 0.5 g magnesium sulfate, 1 g potassium phosphate monobasic, 5 g ammonium sulfate, 500 μg boric acid, 40 μg copper sulfate, 100 μg potassium iodide, 200 μg ferric chloride, 400 μg manganese sulfate, 200 μg sodium molybdate, 400 μg zinc sulfate, 1 μg biotin, 200 μg calcium pantothenate, 1 μg folic acid, 1 mg inositol, 200 μg niacin, 100 μg *p*-aminobenzoic acid, 200 μg pyridoxine, 100 μg riboflavin, 200 μg thiamine, and 0.08% glucose.

Medium is prepared in 10 l quantities, mixed thoroughly, and filter sterilized into an autoclaved glass carboy. Carboy has an outlet port at bottom, leading to a small piece of tubing with a luer lock connector at the end. All entry and exit ports are covered with foil before autoclaving. Outflow tubing is sealed with a metal clamp before filling. Carboy is placed on a shelf above chemostat area.

7.2. Chemostat preparation

A glass-blown chemostat apparatus (Reeves Glass) is outfitted with input tubing including an in-line segment of peristaltic pump tubing and an appropriate luer fitting for connection to the media supply. The overflow port is connected to tubing leading through a bored cork to an effluent collection bottle which drains into a larger reservoir. All free tubing ends are foil-wrapped, and the entire assembly is placed in a tray and autoclaved.

7.3. Chemostat assembly

Autoclaved elements are assembled using sterile technique. Airflow is provided by an aquarium pump, via a water diffuser for humidification, and sterilized by two in-line autoclaved filters. Temperature control at 30 °C is provided by a circulating waterbath attachment to the water jacket of the chemostat vessel.

Once assembled, carboy outflow is connected to chemostat inflow and the tubing is unclamped to allow chemostat to fill by gravity flow. When chemostat begins to overflow (working volume ~200 ml), flow is clamped off via loading of the pump tubing into the peristaltic pump head. The pump has been precalibrated to supply a dilution rate of 0.17 chemostat volumes per hour.

7.4. Inoculation

The strain FY4, a prototroph haploid of the S288C background, is streaked for single colonies from a glycerol stock to a YPD plate and grown at 30 °C for 2 days. A single colony is inoculated into 2.5 ml of glucose-limited chemostat medium and grown overnight at 30 °C. One milliliter of the culture is used to inoculate the chemostat and 1 ml is frozen in glycerol stock as the time 0 sample. Chemostat is grown to saturation overnight and then the pump is started.

7.5. Daily sampling

Daily, the effluent volume is measured and any necessary modification is made to the pump settings. The cork is removed from the effluent bottle and placed in a small tube to passively collect 10 ml culture. One milliliter is frozen in glycerol stock. The sample is measured for A_{600} and Klett density, then briefly sonicated for cell counting in a hemacytometer. Diluted samples are plated to YPD and minimal media agar plates. Notes are also recorded about cell morphology, colony morphology, and chemostat vessel observations (e.g., wall growth, aroma). Carboy supply is monitored and new carboys of sterile media are supplied as necessary. A 10 ml sample from each retired bottle is collected and frozen for analysis of media composition.

In the first 2–3 days after inoculation, the culture has not yet reached steady state. Steady state is usually defined operationally as occurring once all measurements have been equal for 2 days in a row. This should occur at approximately generation 10–15.

7.6. Weekly sampling

Once or twice a week, 25 ml is passively collected from the effluent port, pelleted, and resuspended in glycerol stock for later DNA preparation. Ten milliliters of culture is removed from the main vessel via a port in the

chemostat lid using a sterile pipette. This sample is collected on a filter and snap-frozen for later RNA preparation. Filtrate from the RNA collection is frozen for later metabolite and residual nutrient analysis.

Sampling continues until an error occurs, or the culture develops a clumping phenotype, as defined by clumps that cannot be broken up by light sonication and are observed in most microscope fields.

7.7. Analysis

Data from the experiment are recorded in the database and the raw data index cards are filed in the master system.

Collected samples are processed for DNA and RNA and assayed via microarrays and/or Solexa sequencing for genotype and phenotype differences. Collected media and filtrate samples are analyzed for limiting nutrient concentrations to ensure constant nutrient source and to detect increased consumption.

Representative clones are isolated from population glycerol stocks and assayed for growth phenotypes and mutations. Clones are regrown in new chemostats just until reaching steady state, and then harvested for expression analysis versus the ancestral strain grown in the same conditions.

Once mutations are discovered, their gross frequency can be retrospectively assayed by performing PCR directly on small samples of cells obtained from the population glycerol stocks. The mixed PCR product is sequenced to determine the relative amount of each allele. Time 0 samples are included to ensure mutation was not already present in the inoculum.

Clones may also be subjected to competition versus a marked wild-type ancestor strain. In this case, both strains are grown to steady state in individual chemostats and then mixed. In the null exception, 50% of each strain should be present, but this often gives insufficient survey time for evolved strains with 5–50% fitness increases. When the strain is known or suspected to carry such an advantage, more useful data can be collected using a starting frequency of 5–10%.

8. CONCLUSIONS

Experimental testing of evolutionary questions is almost as old as evolutionary theory itself. The use of these techniques in yeast is yielding exciting results in evolutionary genomics, systems biology, and theory, complementing the excellent comparative genomic and ecological tools also maturing in yeast concurrently. The use of evolution as a tool will also help tune synthetic systems and generate new and useful strains and constructs. This guide is only to be taken as touching on the highlights of this exciting field, and, hopefully, lowering the bar to entry for new researchers.

ACKNOWLEDGMENTS

The author thanks Frank Rosenzweig for being the source of much of her chemostat training and techniques. She also thanks Matt Brauer, David Botstein, and other members of the Dunham and Botstein labs for helping develop these methods. Finally, thanks to the other experimental evolution laboratories who contributed equipment recommendations found in [Table 19.1](#). Please forgive omissions in citations.

REFERENCES

- Adams, J. (2004). Microbial evolution in laboratory environments. *Res. Microbiol.* **155**, 311–318.
- Baganz, F., Hayes, A., Marren, D., Gardner, D. C., and Oliver, S. G. (1997). Suitability of replacement markers for functional analysis studies in *Saccharomyces cerevisiae*. *Yeast* **13**, 1563–1573.
- Blanc, V. M., and Adams, J. (2003). Evolution in *Saccharomyces cerevisiae*: Identification of mutations increasing fitness in laboratory populations. *Genetics* **165**, 975–983.
- Brauer, M. J., Christianson, C. M., Pai, D. A., and Dunham, M. J. (2006). Mapping novel traits by array-assisted bulk segregant analysis in *Saccharomyces cerevisiae*. *Genetics* **173**, 1813–1816.
- Brauer, M. J., Huttenhower, C., Airoidi, E. M., Rosenstein, R., Matese, J. C., Gresham, D., Boer, V. M., Troyanskaya, O. G., and Botstein, D. (2008). Coordination of growth rate, cell cycle, stress response, and metabolic activity in yeast. *Mol. Biol. Cell* **19**, 352–367.
- Brown, C. J., Todd, K. M., and Rosenzweig, R. F. (1998). Multiple duplications of yeast hexose transport genes in response to selection in a glucose-limited environment. *Mol. Biol. Evol.* **15**, 931–942.
- Bryson, V., and Szybalski, W. (1952). Microbial selection. *Science* **116**, 45–51.
- Buckling, A., Craig Maclean, R., Brockhurst, M. A., and Colegrave, N. (2009). The Beagle in a bottle. *Nature* **457**, 824–829.
- Burke, M. K., and Rose, M. R. (2009). Experimental evolution with *Drosophila*. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **296**, R1847–R1854.
- Cowen, L. E., Sanglard, D., Calabrese, D., Sirjusingh, C., Anderson, J. B., and Kohn, L. M. (2000). Evolution of drug resistance in experimental populations of *Candida albicans*. *J. Bacteriol.* **182**, 1515–1522.
- de Kock, S. H., du Preez, J. C., and Kilian, S. G. (2000). The effect of vitamins and amino acids on glucose uptake in aerobic chemostat cultures of three *Saccharomyces cerevisiae* strains. *Syst. Appl. Microbiol.* **23**, 41–46.
- Desai, M. M., Fisher, D. S., and Murray, A. W. (2007). The speed of evolution and maintenance of variation in asexual populations. *Curr. Biol.* **17**, 385–394.
- Dunham, M. J., Badrane, H., Ferea, T., Adams, J., Brown, P. O., Rosenzweig, F., and Botstein, D. (2002). Characteristic genome rearrangements in experimental evolution of *Saccharomyces cerevisiae*. *Proc. Natl. Acad. Sci. USA* **99**, 16144–16149.
- Dykhuizen, D. E., and Hartl, D. L. (1983). Selection in chemostats. *Microbiol. Rev.* **47**, 150–168.
- Elena, S. F., and Lenski, R. E. (2003). Evolution experiments with microorganisms: The dynamics and genetic bases of adaptation. *Nat. Rev. Genet.* **4**, 457–469.
- Ferea, T. L., Botstein, D., Brown, P. O., and Rosenzweig, R. F. (1999). Systematic changes in gene expression patterns following adaptive evolution in yeast. *Proc. Natl. Acad. Sci. USA* **96**, 9721–9726.

- Francis, J. C., and Hansche, P. E. (1972). Directed evolution of metabolic pathways in microbial populations. I. Modification of the acid phosphatase pH optimum in *S. cerevisiae*. *Genetics* **70**, 59–73.
- Garland, T., and Kelly, S. A. (2006). Phenotypic plasticity and experimental evolution. *J. Exp. Biol.* **209**, 2344–2361.
- Gresham, D., Ruderfer, D. M., Pratt, S. C., Schacherer, J., Dunham, M. J., Botstein, D., and Kruglyak, L. (2006). Genome-wide detection of polymorphisms at nucleotide resolution with a single DNA microarray. *Science* **311**, 1932–1936.
- Gresham, D., Dunham, M. J., and Botstein, D. (2008). Comparing whole genomes using DNA microarrays. *Nat. Rev. Genet.* **9**, 291–302.
- Gresham, D., Desai, M. M., Tucker, C. M., Jenq, H. T., Pai, D. A., Ward, A., DeSevo, C. G., Botstein, D., and Dunham, M. J. (2008). The repertoire and dynamics of evolutionary adaptations to controlled nutrient-limited environments in yeast. *PLoS Genet.* **4**, e1000303.
- Groisman, A., Lobo, C., Cho, H., Campbell, J. K., Dufour, Y. S., Stevens, A. M., and Levchenko, A. (2005). A microfluidic chemostat for experiments with bacterial and yeast cells. *Nat. Methods* **2**, 685–689.
- Hansche, P. E., Beres, V., and Lange, P. (1978). Gene duplication in *Saccharomyces cerevisiae*. *Genetics* **88**, 673–687.
- Hayes, A., Zhang, N., Wu, J. Y., Butler, P. R., Hauser, N. C., Hoheisel, J. D., Lim, F. L., Sharrocks, A. D., and Oliver, S. G. (2002). Hybridization array technology coupled with chemostat culture: Tools to interrogate gene expression in *Saccharomyces cerevisiae*. *Methods* **26**, 281–290.
- Hess, D. C., Lu, W., Rabinowitz, J. D., and Botstein, D. (2006). Ammonium toxicity and potassium limitation in yeast. *PLoS Biol.* **4**, e351.
- Kao, K. C., and Sherlock, G. (2008). Molecular characterization of clonal interference during adaptive evolution in asexual populations of *Saccharomyces cerevisiae*. *Nat. Genet.* **40**, 1499–1504.
- Kasemets, K., Drews, M., Nisamedtinov, I., Adamberg, K., and Paalme, T. (2003). Modification of A-stat for the characterization of microorganisms. *J. Microbiol. Methods* **55**, 187–200.
- Kuthan, M., Devaux, F., Janderova, B., Slaninova, I., Jacq, C., and Palkova, Z. (2003). Domestication of wild *Saccharomyces cerevisiae* is accompanied by changes in gene expression and colony morphology. *Mol. Microbiol.* **47**, 745–754.
- Lane, P. G., Oliver, S. G., and Butler, P. R. (1999). Analysis of a continuous-culture technique for the selection of mutants tolerant to extreme. *Biotechnol. Bioeng.* **65**, 397–406.
- Lang, G. I., Murray, A. W., and Botstein, D. (2009). The cost of gene expression underlies a fitness trade-off in yeast. *Proc. Natl. Acad. Sci. USA* **106**, 5755–5760.
- Leu, J. Y., and Murray, A. W. (2006). Experimental evolution of mating discrimination in budding yeast. *Curr. Biol.* **16**, 280–286.
- Mark, G. H., Davey, C. L., and Kell, D. B. (1991). The permittistat: A novel type of turbidostat. *J. Gen. Microbiol.* **137**, 735.
- Monod, J. (1950). La Technique de culture continue. Theorie et applications. *Ann. Inst. Pasteur* **79**, 390–410.
- Norris, J. R., and Ribbons, D. W. (1970). Turbidostats. *Methods Microbiol.* **2**, 349–376.
- Novick, A., and Szilard, L. (1950a). Description of the chemostat. *Science* 715–716.
- Novick, A., and Szilard, L. (1950b). Experiments with the chemostat on spontaneous mutations of bacteria. *Proc. Natl. Acad. Sci. USA* **36**, 708–719.
- Paquin, C., and Adams, J. (1983a). Relative fitness can decrease in evolving asexual populations of *S. cerevisiae*. *Nature* **306**, 368–371.

- Paquin, C., and Adams, J. (1983b). Frequency of fixation of adaptive mutations is higher in evolving diploid than haploid yeast populations. *Nature* **302**, 495–500.
- Philippe, N., Crozat, E., Lenski, R. E., and Schneider, D. (2007). Evolution of global regulatory networks during a long-term experiment with *Escherichia coli*. *Bioessays* **29**, 846–860.
- Saito, H., and Inoue, T. (2007). RNA and RNP as new molecular parts in synthetic biology. *J. Biotechnol.* **132**, 1–7.
- Schuller, D., and Casal, M. (2005). The use of genetically modified *Saccharomyces cerevisiae* strains in the wine industry. *Appl. Microbiol. Biotechnol.* **68**, 292–304.
- Segre, A. V., Murray, A. W., and Leu, J. Y. (2006). High-resolution mutation mapping reveals parallel experimental evolution in yeast. *PLoS Biol.* **4**, e256.
- Shou, W., Ram, S., and Vilar, J. M. G. (2007). Synthetic cooperation in engineered yeast populations. *Proc. Natl. Acad. Sci. USA* **104**, 1877–1882.
- Thompson, D. A., Desai, M. M., and Murray, A. W. (2006). Ploidy controls the success of mutators and nature of mutations during budding yeast evolution. *Curr. Biol.* **16**, 1581–1590.
- Torres, E. M., Sokolsky, T., Tucker, C. M., Chan, L. Y., Boselli, M., Dunham, M. J., and Amon, A. (2007). Effects of aneuploidy on cellular physiology and cell division in haploid yeast. *Science* **317**, 916–924.
- van Dijken, J. P., Bauer, J., Brambilla, L., Duboc, P., Francois, J. M., Gancedo, C., Giuseppin, M. L., Heijnen, J. J., Hoare, M., Lange, H. C., Madden, E. A., Niederberger, P., *et al.* (2000). An interlaboratory comparison of physiological and genetic properties of four *Saccharomyces cerevisiae* strains. *Enzyme Microb. Technol.* **26**, 706–714.
- Verstrepen, K. J., Chambers, P. J., and Pretorius, I. S. (2006). The development of superior yeast strains for the food and beverage industries: Challenges, opportunities and potential benefits. In “Yeasts in Food and Beverages,” (A. Querol and G. H. Fleet, eds.), Springer-Verlag, Berlin Heidelberg.
- Zeyl, C. (2004). Experimental studies on ploidy evolution in yeast. *FEMS Microbiol. Lett.* **233**, 187–192.
- Zeyl, C. (2005). The number of mutations selected during adaptation in a laboratory population of *Saccharomyces cerevisiae*. *Genetics* **169**, 1825–1831.
- Zeyl, C. (2006). Experimental evolution with yeast. *FEMS Yeast Res.* **6**, 685–691.
- Zeyl, C., and DeVisser, J. A. (2001). Estimates of the rate and distribution of fitness effects of spontaneous mutation in *Saccharomyces cerevisiae*. *Genetics* **157**, 53–61.