

# A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly

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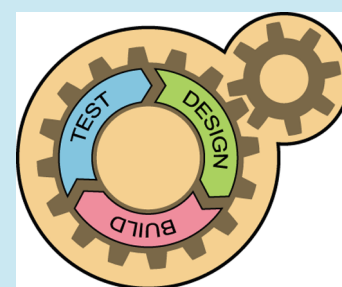
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## S Supporting Information

**ABSTRACT:** *Saccharomyces cerevisiae* is an increasingly attractive host for synthetic biology because of its long history in industrial fermentations. However, until recently, most synthetic biology systems have focused on bacteria. While there is a wealth of resources and literature about the biology of yeast, it can be daunting to navigate and extract the tools needed for engineering applications. Here we present a versatile engineering platform for yeast, which contains both a rapid, modular assembly method and a basic set of characterized parts. This platform provides a framework in which to create new designs, as well as data on promoters, terminators, degradation tags, and copy number to inform those designs. Additionally, we describe genome-editing tools for making modifications directly to the yeast chromosomes, which we find preferable to plasmids due to reduced variability in expression. With this toolkit, we strive to simplify the process of engineering yeast by standardizing the physical manipulations and suggesting best practices that together will enable more straightforward translation of materials and data from one group to another. Additionally, by relieving researchers of the burden of technical details, they can focus on higher-level aspects of experimental design.

**KEYWORDS:** yeast, toolkit, characterized parts, golden gate, MoClo



Synthetic biology is driven by the desire to engineer novel biological functions that push the boundaries of what can be accomplished within living cells. Unfortunately, the potential power of the cell also brings with it a level of complexity that makes engineering biological systems extremely difficult. Synthetic biologists have sought ways to abstract the layers of complexity into components with predictable interactions, making it more feasible to undertake large engineering projects. Despite these efforts, the inner workings of the cell continue to elude understanding, and while certain elements can be highly predictable, the system behavior as a whole is difficult to anticipate. These challenges have led to an additional, and equally important, aspect to synthetic biology: rapid prototyping.<sup>1–4</sup> Because manipulations to the cell often lead to unexpected results, progress is best made by rapidly iterating through highly parallelized experiments to explore a wide parameter space.<sup>5,6</sup> It is the combination of these two principles—predictable parts and rapid prototyping—that give synthetic biologists the ability to approach difficult problems in energy,<sup>7,8</sup> agriculture,<sup>9</sup> and human health.<sup>10–12</sup> *Saccharomyces cerevisiae* is growing in popularity as a chassis for synthetic biology due to its powerful genetic tools,<sup>13–15</sup> extensively studied biology,<sup>16–19</sup> and long history of industrial applications.<sup>20–22</sup> In this work, we present a synthetic biology toolkit for engineering yeast that simplifies and accelerates experimentation in this important model organism.

Abstraction is a fundamental principle in any engineering discipline. It allows an engineer to focus on an individual component with the assurance that it will interface correctly with other components, both existing and future. When applied to synthetic biology, abstraction typically refers to the level of complexity of the DNA that is being built or introduced into cells. “Parts” are often thought of as one of the most basic DNA sequence elements that can be assigned a function. For example, a coding sequence, a transcriptional terminator, and an origin of replication could all be described as parts. Although these parts can be broken down further—they contain, among other things, a start and stop codon, a hairpin, and a protein binding site, respectively—the benefit of abstraction is the ability to ignore those lower level details and work with a part based solely on its reported function. Extensive efforts by others in the field have contributed to the Registry of Standard Biological Parts, a catalog of DNA sequences and characterization data that continues to grow each year (<http://partsregistry.org>).<sup>23</sup> The Registry, however, is notably biased toward working in bacterial systems, particularly *Escherichia coli*, and with growing interest in yeast as a synthetic biology host, it is becoming apparent that the field needs a more extensive set of standard yeast parts. For this toolkit, we collected,

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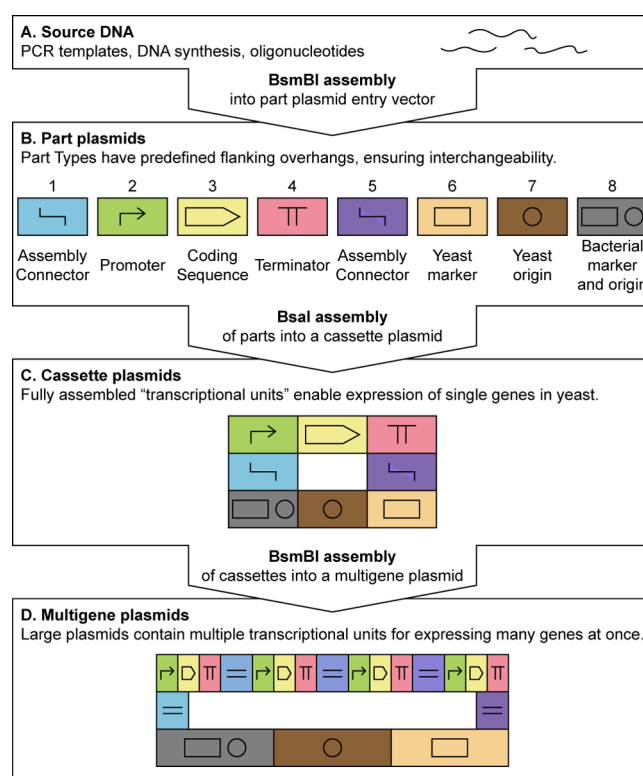
constructed, and characterized a starter set of useful parts to lay the foundation for a standardized engineering platform, and these parts are available from Addgene.

Prototyping is a more necessary step in synthetic biology than in other engineering fields, as synthetic biologists lack the ability to accurately predict behavior, even of devices made from parts of known function.<sup>24–26</sup> When working in fast-growing cells such as yeast, cloning is often the bottleneck step in an experimental cycle. The lag between having a DNA design and actually obtaining the physical DNA is far too long to support a robust prototyping workflow. The solution that many groups have developed is standardization of cloning.<sup>27–32</sup> For example, the BioBrick standard (and its relatives) defines a set of restriction enzyme sites that are used to flank each part in a vector.<sup>27,30</sup> When those restriction enzymes are used to join two parts, the junction contains an assembly “scar”, and the resulting plasmid reconstitutes the sites external to the newly combined parts (an idempotent operation). This enables an endless number of cycles of pairwise assembly. More recently, Golden Gate assembly based methods have increased in popularity due to the added flexibility provided by the use of Type II restriction enzymes, which cut outside their recognition sequence and provide unique cohesive ends to enable directional, multi-insert, one-pot cloning.<sup>33</sup> One example is the MoClo (modular cloning) system, which categorizes parts as “types” based on their function and location in a completed device (e.g., promoter types or coding sequence types) and designates particular overhangs that flank each type, allowing all parts of a particular type to be interchangeable.<sup>32</sup> In this work, we adapted the MoClo strategy specifically to build yeast expression devices. The major advantage of using a standardized system such as MoClo is that once parts are constructed, they are immediately available for incorporation into devices and no longer require synthesis of oligonucleotides, PCR amplification and purification, or verification by sequencing. This allows us to construct from parts, a plasmid carrying multiple gene expression devices in as little as 2 days.

## RESULTS AND DISCUSSION

**Definition of an Assembly Standard for Yeast.** Our standard for assembling DNA for expression in yeast is a bottom-up hierarchical approach to DNA construction (Figure 1). A description of the assembly scheme, part types, and overhang sequences are discussed briefly here and in more detail in the Supporting Information. For brevity, Golden Gate assemblies using either BsaI or BsmBI are referred to as “BsaI assembly” and “BsmBI assembly”.

Our workflow for assembling complex plasmids for expressing multiple genes in yeast has multiple steps that correspond to our abstraction layers. First, source DNA is obtained through PCR, synthesis or another user-preferred method. That source DNA is “domesticated” via BsmBI assembly into a universal entry vector, resulting in a “part” plasmid. Part plasmids come in different Types, numbered 1 through 8 (with some optional subtypes). Each part Type is defined by the sequences of the upstream and downstream flanking overhangs generated when digested by BsaI. All parts of a particular Type are interchangeable, which lends the system well to combinatorial experiments. Part plasmids are joined in a BsaI assembly to form a “cassette” plasmid that, in most cases, is used to express a single gene in yeast (a transcriptional unit, TU, comprised of a promoter, coding sequence, and terminator). These cassettes can optionally be

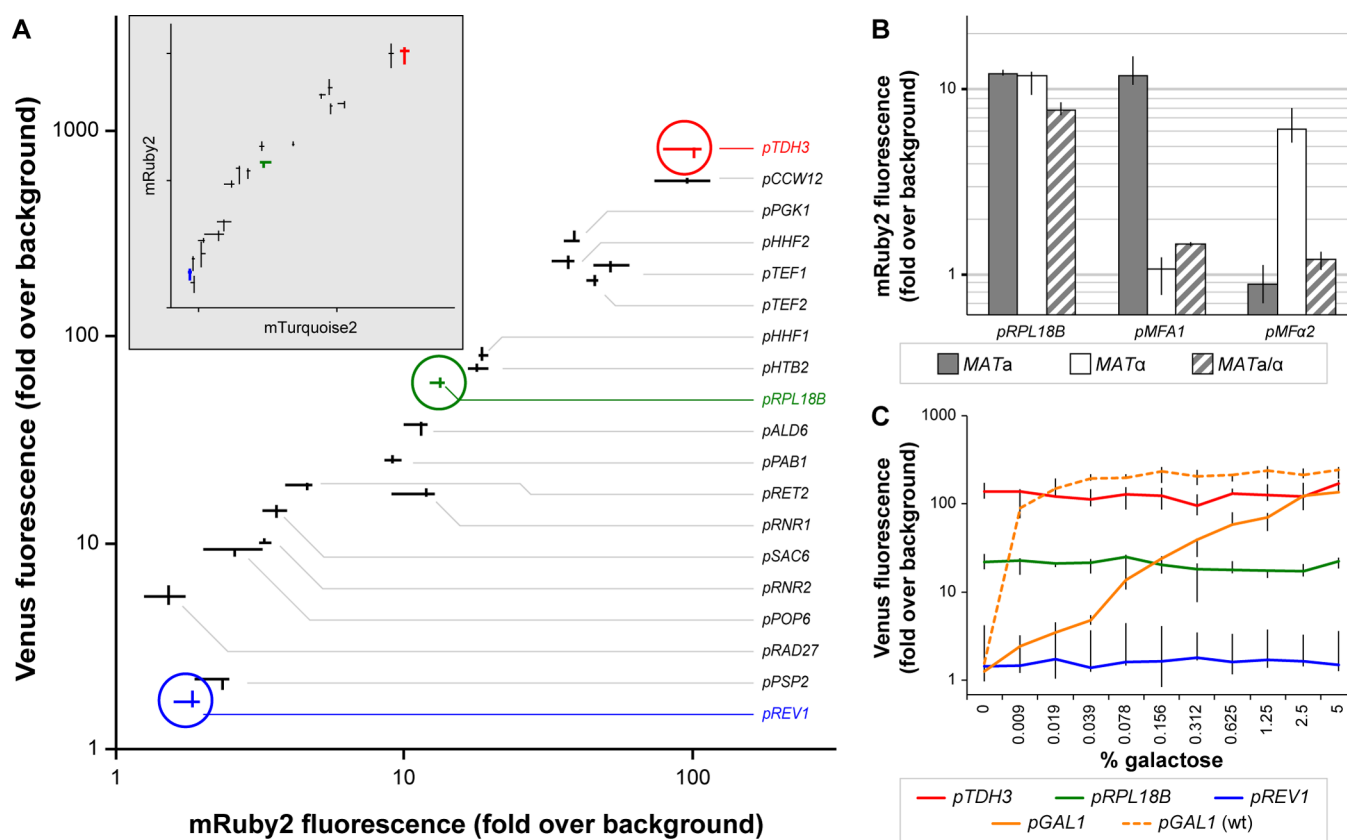


**Figure 1.** Standardized, hierarchical assembly strategy based on MoClo. (A) Source DNA is obtained *via* PCR, DNA synthesis, or oligonucleotides, then assembled using BsmBI into a part plasmid entry vector. (B) Part plasmids of a particular Type have unique upstream and downstream BsaI-generated overhangs. All part plasmids of the same Type are therefore interchangeable. Plasmids at this stage typically confer chloramphenicol resistance in *E. coli*. One part plasmid of each Type is assembled using BsaI to form a cassette plasmid. (C) Cassette plasmids contain a complete transcriptional unit (TU), and can be transformed directly into yeast. Plasmids at this stage typically confer ampicillin resistance in *E. coli*. Alternatively, cassette plasmids can be further assembled using BsmBI to form a multigene plasmid. (D) Multigene plasmids contain multiple TUs, the order of which is dictated by the Assembly Connector parts used to flank the individual cassettes. Plasmids at this stage typically confer kanamycin resistance in *E. coli*.

joined in a final BsmBI assembly to form “multi-gene” plasmids that, as the name suggests, are used to simultaneously express multiple genes. The multigene assembly is enabled by the use of Assembly Connectors (Type 1 and 5) that, in similar fashion to each part plasmid’s unique BsaI overhangs, contain unique BsmBI overhangs that flank each cassette. At each round of assembly, the antibiotic selection is changed to minimize background (typically, chloramphenicol → ampicillin → kanamycin). Using this workflow, we can construct a multigene plasmid from PCR templates in only 3 days. This construction time is typically reduced to only 2 days, since, in most cases, the final multigene plasmids are built from existing parts.

There are many benefits to the standard we defined, which should prove useful to synthetic biologists with a wide range of needs. First, the cloning protocols are extremely simple, requiring no PCR amplification or purification steps after the initial part creation. Second, the standardized Golden Gate assemblies are highly robust. It was previously shown that for a 10-part assembly with an optimized set of overhangs, 97% of isolated transformants contained a correctly assembled





**Figure 3.** Characterization of promoters. (A) The relative strength of 19 constitutive promoters is consistent across two coding sequences, mRuby2 and Venus. Three promoters (strong *pTDH3*, medium *pRPL18B*, and weak *pREV1*) that are used throughout this work are highlighted. The horizontal and vertical bars represent the range of four biological replicates, and the intersection represents the median value. (inset) A third fluorescent protein, mTurquoise2, was also tested, and a larger plot can be found in Supporting Figure S1. (B) The mating-type-specific promoter, *pMFA1*, is only active in the *MATa* haploid; *pMFa2* is only active in *MATα* haploids; neither promoter is active in the opposite haploid or in the diploid. The expression level of *pRPL18B* in the three strains is shown for reference. The height of the bars represents the median value of four biological replicates, and the error bars show the range. (C) Galactose induction of *pGAL1* increases expression from background levels up to the highest expressing constitutive promoter, *pTDH3*. All solid line data were collected from a  $\Delta gal2$  strain. The dashed line shows a much more sensitive response to galactose induction in a wild type strain. Points represent the median value of four biological replicates, and error bars show the range.

collection of 96 parts compatible with this standard for efficiently engineering yeast strains (Figure 2 and Supporting Table S1). This starter collection contains an assortment of promoters, terminators, fluorescent proteins, peptide tags, selectable markers, and origins of replication, as well as a part entry vector into which new parts can be cloned. Additionally, we have included sequences targeting chromosomal loci for integration, and genome-editing tools for introducing double-strand breaks to stimulate homologous recombination. Finally, rather than provide a large array of different vectors, the assembly standard enables construction of custom vectors directly from parts in the toolkit, and one such vector is included as an example (Supporting Information).

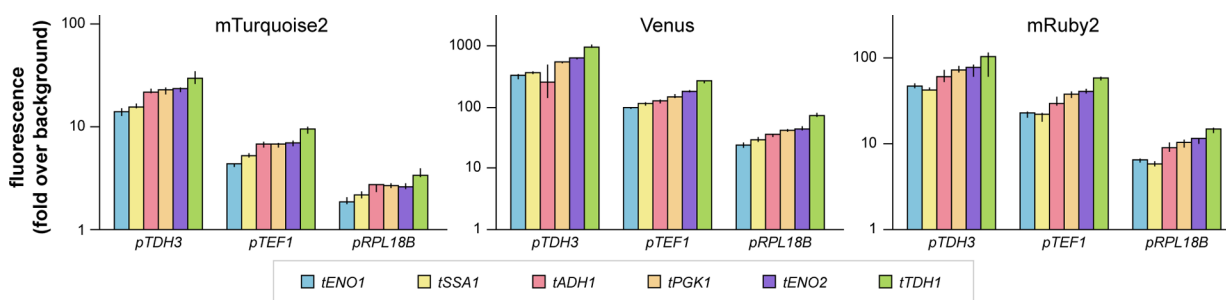
**Characterization of Promoters.** We have characterized 19 constitutive promoters, two mating-type-specific promoters, and two inducible promoters, all cloned from the yeast genome (although synthetic promoters<sup>41,42</sup> could easily be ported into the system as well). The promoters were selected to span a wide range of transcriptional strengths while minimizing variability between growth conditions.<sup>43</sup> In general, they constitute the 700 bp directly upstream of the native start codon, although in some cases where another ORF was less than 700 bp away, we cloned only the intergenic noncoding region. To examine the strength of each promoter, we cloned it

upstream of a fluorescent reporter (mRuby2, Venus, and mTurquoise2) and measured bulk fluorescence on a plate reader.

It was previously shown that the strength of constitutive promoters cloned from the yeast genome was largely independent of the downstream coding sequence,<sup>44</sup> an important distinction between controlling expression in bacteria and yeast. This held true for the 19 constitutive promoters characterized in this work (which include some overlap with Lee *et al.*, 2013)<sup>44</sup> (Figure 3A and Supporting Figure S1). The promoters span a range of up to 3 orders of magnitude, and there are also some promoters that have very similar expression strengths, allowing them to be interchangeable so as to reduce the risk of undesired homologous recombination in multigene plasmids due to repeated sequences. Although we only tested these promoters in one type of media, the majority of native yeast promoters have been shown to maintain their relative expression strengths in different growth conditions, although the absolute strengths may change.<sup>45</sup>

It is sometimes useful to have genes under dynamic control, and for this we provide two tools: mating-type-specific and inducible promoters. We tested *pMFA1* and *pMFa2* and found that they have very close to background levels of fluorescence





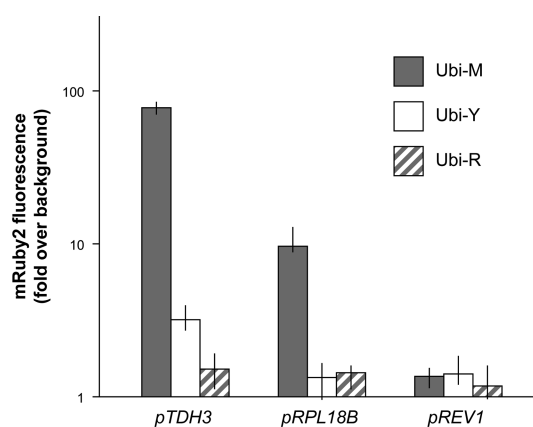
**Figure 4.** Characterization of terminators. Six terminators were cloned behind three fluorescent proteins, each driven by three promoters. The relative expression levels for this set of terminators are largely independent of the coding sequence and the promoter. The height of the bars represents the median value of four biological replicates, and the error bars show the range.

in both the opposite mating-type haploid and diploid strains and a 6- to 10-fold induction in the appropriate haploid (Figure 3B). We also tested *pGAL1* in varying concentrations of galactose and observed a 100-fold induction (Figure 3C). Although the promoter can be used in wild-type strains, the response is very sensitive to low concentrations of galactose; a strain with the *GAL2* transporter knocked out should be used for more graded control overexpression.<sup>46</sup> Finally, we tested *pCUP1* in varying concentrations of copper(II) sulfate ( $\text{CuSO}_4$ ) and observed a 55-fold induction (Supporting Figure S2). This promoter exhibits leaky expression under basal conditions, with approximately 7-fold fluorescence over background when  $\text{CuSO}_4$  is not added to the media. This may be due in part to the  $\text{CuSO}_4$  that is present at 250 nM in the yeast nitrogen base commonly used to make defined media.

**Characterization of Terminators.** The impact of different transcriptional terminators on gene expression can vary considerably, and could provide a secondary mode of control to complement the promoters. However, for simplicity, we opted in this toolkit to provide terminators that yielded approximately the same expression output. Using expression data from the whole yeast genome,<sup>43</sup> we selected six of the most highly expressed genes and cloned the 225 bp immediately downstream of the stop codon. We assembled these terminators with each of our three fluorescent reporters and each using three promoters. The largest difference in expression we observed between terminators for a given promoter and fluorescent protein was 3.6-fold (Figure 4). In general, the fold-changes produced by different promoters were greater than those effected by the terminators, but this was not always the case. If applications are sensitive to small fold-changes of expression, we advise characterizing individual promoter-terminator pairs to ensure that the desired levels of expression are obtained.

**Protein Degradation Tags.** In addition to controlling transcript levels, protein levels can be tuned by fusing degradation tags to the N-terminus. We have included three such tags of varying strengths, Ubi-M (weak), Ubi-Y (medium), and Ubi-R (strong), which can be used to adjust the rate of protein turnover.<sup>47</sup> We fused these tags to the N-terminus of mRuby2, and expressed them using a strong, moderate, and weak promoter, *pTDH3*, *pRPL18B*, and *pREV1*, respectively. The strong degradation tag (Ubi-R) resulted in no detectable fluorescence at any expression level, while the medium strength degradation tag (Ubi-Y) resulted in detectable levels of fluorescence at only the highest expression level (Figure 5).

**Copy Number, Gene Expression, and Single-Cell Variability.** When engineering yeast strains expressing multi-

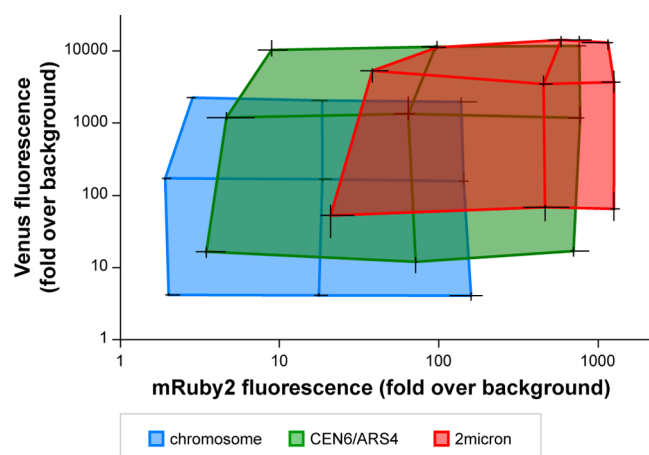


**Figure 5.** Protein degradation tags. Three N-terminal degradation tags were fused to mRuby2 and expressed using three different promoters. Steady-state fluorescence levels are dependent on the difference between the strength of the promoter and the strength of the degradation tag. The height of the bars represents the median value of six biological replicates, and the error bars show the range.

ple heterologous proteins, it is important to consider the relative expression of those proteins. As described above, protein levels can be controlled by changing promoters, terminators, or degradation rates. However, another important consideration is the copy number of the gene(s). Typically, one of three systems is used to express genes in yeast: single-copy integrations into the chromosome, low-copy CEN6/ARS4 plasmids, and high-copy 2micron plasmids. One could easily assume that the differences in copy number simply titrate gene expression accordingly, but we observed that there are subtle, but important, effects that could influence the decision to use one system over another.

We cloned cassettes expressing either mRuby2 or Venus under strong, moderate, and weak promoters (*pTDH3*, *pRPL18B*, and *pREV1*, respectively). Versions of these cassettes were made for each of the three copy numbers. Finally, each of the nine possible combinations of the three promoters and two genes were either assembled in tandem onto a single chromosomal locus/plasmid or kept separate in two loci/plasmids. We measured bulk fluorescence of both fluorescent proteins to compare protein expression levels of the cell populations at the three copy-numbers (Figure 6 and Supporting Figure S3).

In the chromosomally integrated strains, the different promoter combinations fill out the points of a regular grid, as expected. In the low-copy CEN6/ARS4 plasmid system, the absolute fluorescence is generally higher compared to the

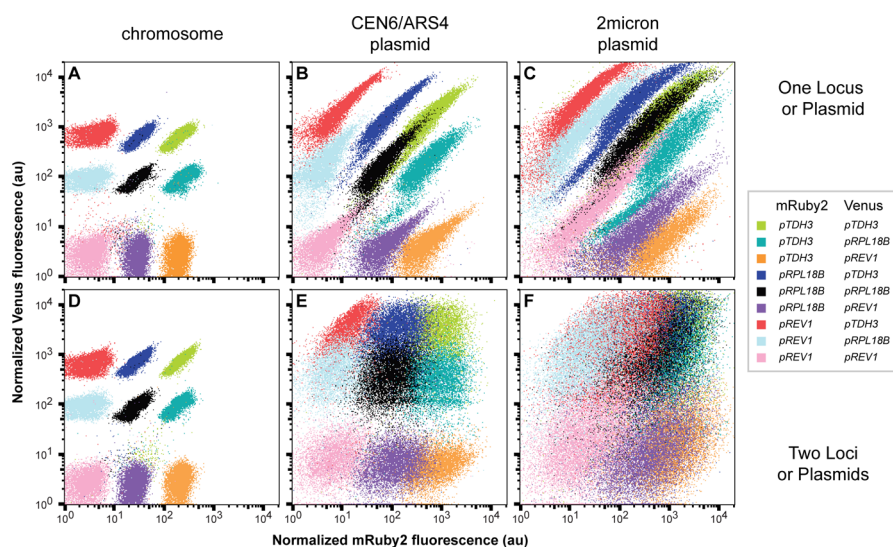


**Figure 6.** Effect of copy number on gene expression. Three promoters (*pTDH3*, *pRPL18B*, and *pREV1*) drive two fluorescent proteins (mRuby2 and Venus) in all nine possible combinations. These nine combinations are integrated into the chromosome (blue), expressed from a low-copy plasmid (green), and expressed from a high-copy plasmid (red). The translucent, shaded boxes show the range of expression spanned by each respective copy number. For lower strength promoters, increasing copy number gives higher fluorescence; but for the strongest promoter, there is a much smaller difference between the low- and high-copy plasmids. Each gene is integrated in a separate locus or expressed from a separate plasmid. The horizontal and vertical bars represent the range of four biological replicates, and the intersection represents the median value.

chromosome, again, as expected. Interestingly, the range between the highest and lowest expression is actually slightly greater in the CEN6/ARS4 plasmid system. Compared to low-copy plasmids, the high-copy 2micron plasmids showed considerably more irregular expression patterns. In the two-plasmid, 2micron system, the grid is preserved, but compressed at higher expression levels, suggesting that some expression

machinery in the cell is limiting and that having more copies of the DNA has little effect on increasing expression; *i.e.*, the average fluorescence of cells with the strongest promoter is similar between low- and high-copy plasmids. In the single-plasmid, 2micron system, not only is the grid compressed, but also it appears that high expression of one gene seems to reduce the expression of the second gene (Supporting Figure S3). On the basis of flow cytometry, there appears to be a bimodal distribution for some of these populations (Figure 7C, *e.g.*, *pTDH3*-mRuby2/*pRPL18B*-Venus), which is consistent with previous studies comparing the distribution of expression in 2micron and chromosomally integrated systems.<sup>48</sup> Interestingly, this effect is not nearly as pronounced in the chromosome or on the low-copy plasmid. It is unclear why this would be specific to the high-copy plasmid. On the basis of these data, we believe that use of high-copy 2micron plasmids should generally be avoided, since the highest expression levels accessible by them are very nearly accessible by low-copy CEN6/ARS4 plasmids, and low-copy plasmids give greater access to lower expression, and in general have less erratic expression patterns.

Another parameter we examined was cell-to-cell variability in the relative expression of two genes. While it has been shown that strains expressing fluorescent proteins from chromosomally integrated genes display much tighter distributions compared to those expressing from 2micron plasmids,<sup>48</sup> we were curious about any additional effects of propagating one *versus* multiple plasmids. We took the same cultures used to measure bulk fluorescence and ran them on a flow cytometer to measure single-cell fluorescence of the two fluorescent proteins (Figure 7). As expected, the single-cell measurements revealed that the variability in fluorescence increased considerably when moving from the chromosome to either a low-copy or high-copy plasmid, indicating that the precise copy number of these plasmids is not tightly regulated. When expressed from a single locus/plasmid, the expression of the two fluorescent proteins



**Figure 7.** Effect of copy number on single-cell gene expression. The same strains expressing mRuby2 and Venus that measured for bulk fluorescence in Figure 6 and Supporting Figure S3 were run on a flow cytometer: chromosomally integrated in a single locus (A) or two loci (D); on a single (B) or two (E) low-copy plasmids; on a single (C) or two (F) high-copy plasmids. As copy number increased, the variability of expression also increased. For all single-locus strains, the expression of the two fluorescent proteins was well correlated, suggesting that copy number is the main contributor to variation in expression. When expressed from two plasmids, correlation between fluorescent proteins is lost, suggesting that the copy number of each plasmid is independent of the other. Fluorescence in each channel was normalized for cell size by dividing by forward-scatter. Dot plots for each sample represent 5000–10000 events.

was well correlated, as evidenced by the distribution of each strain along the diagonal. This result suggests that DNA copy number is the primary source of added variation in plasmid-based expression systems, a model which is further supported by the data from two loci/plasmids. Strains expressing mRuby2 and Venus from two separate loci in the chromosome showed distributions that are nearly identical to the single locus, chromosomal strains. In contrast, the low-copy and high-copy plasmids lose their tight correlation between the two fluorescent proteins when the two genes are expressed from separate plasmids. Thus, not only is the copy number of a plasmid highly variable, the relative copy numbers of two plasmids in the same cell are not well correlated. Therefore, we would recommend that genes be integrated into the chromosome whenever possible. If, however, higher expression than can be attained from the chromosome is required, use of a low-copy plasmid is preferred, and all genes should be expressed from the same plasmid rather than split onto multiple plasmids. Accordingly, the assembly standard we provide here accommodates the facile assembly of up to six genes on a single plasmid or in a single chromosomal locus, with more possible if additional Assembly Connectors are designed.

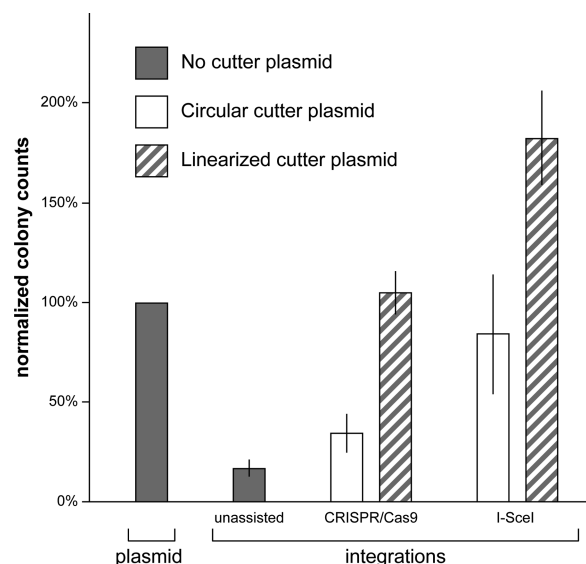
#### High-Efficiency Integrations into the Chromosome.

Yeast is well suited to chromosomal modification due to its efficient homologous recombination machinery. This allows for site-specific integration of DNA into the chromosome by simply transforming linear DNA flanked by sequences homologous to the target locus. However, compared to plasmid transformation, integrations usually result in almost an order of magnitude reduction in colony counts, which is one reason why the use of plasmids is often preferred. Given the desire for chromosomal integrations described earlier, it is evident that a higher efficiency method for integrating into the chromosome is necessary, particularly when working with large libraries. Fortunately, it was previously shown that transformation efficiency could be dramatically improved by using a homing endonuclease to generate a double-strand break in the chromosome and stimulate recombination.<sup>49</sup> More recently, the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated (Cas) system has been used for similar purposes.<sup>50–52</sup> We tested both systems to directly compare their effects on chromosomal library construction.

First, we prepared an experimental strain by integrating a “landing pad” using conventional homologous recombination of linear DNA (Supporting Figure S4A). This landing pad contained an I-SceI recognition site; the I-SceI recognition site conveniently contains an NGG protospacer adjacent motif (PAM) close to the I-SceI cutting site, and we added an extra 10 bp upstream of the site to create a 20 bp targeting sequence for a single guide RNA (sgRNA); we also included a partial *URA3* coding sequence and terminator that by itself is nonfunctional.

Next, we designed the repair DNA we were integrating to contain a Venus-expressing cassette and a *HIS3*-expressing cassette, flanked by homology to the sequence upstream of the landing pad and to the partial *URA3* marker. Thus, when the DNA integrated successfully, the cells would be prototrophic for histidine and uracil, and they would be fluorescent. If the DNA integrated off-target, the cells would be prototrophic for histidine and fluorescent, but they would remain auxotrophic for uracil, allowing us to measure the rate of off-target integration by selecting on 5-fluoro-orotic acid (5-FOA).

Finally, we compared the efficiency of integration when the repair DNA was transformed unassisted, with a transient “cutter” plasmid (we did not select for it) expressing Cas9 and an sgRNA, or with a transient cutter plasmid expressing I-SceI. As a control for cell competency, we transformed a circular version of the repair DNA that also contained an origin of replication (Figure 8 and Supporting Figure S4B). Compared



**Figure 8.** High-efficiency integration into the chromosome. Integration of linear DNA into the chromosome by homologous recombination yields 6-fold fewer colonies (compare shaded bars). Adding in a Cas9 or I-SceI improves transformation efficiency by 2-fold and 5-fold, respectively (compare white bars to unassisted integration). Linearizing the Cas9 or I-SceI expression plasmid prior to transformation further improves transformation efficiency to match plasmid transformation efficiency or exceed it by 1.8-fold, respectively (compare striped bars to plasmid transformation). Colony counts are normalized to the number of colonies from the plasmid transformation for each replicate. The height of the bars represents the mean value of three biological replicates, and the error bars show the standard error of the mean.

to the plasmid transformation, unassisted integration gave approximately 6-fold fewer colonies. When a CRISPR/Cas9 cutter plasmid was cotransformed, there was a 2-fold increase in colony count over unassisted integration; when an I-SceI cutter plasmid was cotransformed, there was an additional 2.5-fold increase (5-fold compared to unassisted). We were able to further improve the efficiency for both the Cas9 and I-SceI systems by linearizing (with a restriction digest) the cutter plasmid prior to transformation. Doing so brought the efficiency of Cas9-assisted integration to match that of plasmid transformation. Incredibly, the linearized I-SceI-expressing DNA actually increased integration efficiency to 1.8-fold over the rate of plasmid transformation. We measured the rate of off-target integration for this most efficient method (linearized I-SceI) and found that only 0.02% of transformants were 5-FOA resistant, and therefore had integrated the repair DNA improperly. By measuring Venus fluorescence, we found that 0.14% of transformants contained multiple integrations. It is unclear why linearizing the cutter plasmid increases the transformation efficiency, but one possibility is that linear DNA enters the cell and/or nucleus more efficiently than circular plasmids. Regardless, the ease with which sequences

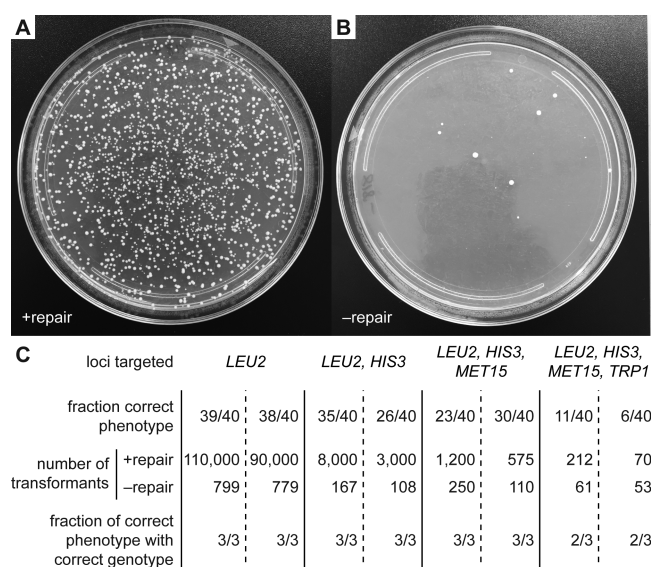


can now be integrated into the chromosome should further encourage the use of integrations over plasmids, even with the high transformation efficiency requirements when working with large libraries.

**Multiplex, Markerless Genome Editing Using CRISPR/Cas9.** Although the high-efficiency integration method described above is very powerful for integrating sequences into the chromosome, it requires a selectable marker be present in the integrated DNA. There are some cases where this requirement is undesirable, such as knocking out multiple genes in a single strain. In this case, a unique marker is needed for each locus; markers must be introduced and then removed for each sequential knockout; or complex mating and screening strategies have to be used to collect all the mutants in a single strain. To avoid these tedious procedures, we adapted the recently described CRISPRm method for making multiple genome edits simultaneously.<sup>51</sup>

First, we designed two sgRNAs to target each of four genes—*LEU2*, *HIS3*, *MET15*, and *TRP1*—and repair DNAs (PCR products with 60 bp of homology flanking a 20 bp barcode, see Supporting Figure S5A, Supporting Table S2, and Supporting Table S3) that target those loci and introduce a premature stop codon. We assembled each sgRNA onto a CEN6/ARS4 plasmid containing a Cas9 expression cassette with a *URA3* marker (Supporting Figure S5B). We then transformed each plasmid with or without its cognate repair DNA and selected for transformants on synthetic media lacking uracil (Figure 9A and 9B, and Supporting Figure S5C). For seven out of eight guides, the transformations with repair DNA had over 100-fold more colonies than those without repair DNA. This large difference in colony yields suggests that when Cas9 successfully targets a locus in the genome, the double-strand break it introduces is toxic to the cell; when a repair DNA is present, it removes the target and abolishes the toxicity caused by Cas9. Thus, selecting for the Cas9 plasmid indirectly selects for repairs to the genome at the targeted locus. It should be noted that both here and elsewhere,<sup>51,52</sup> it has been shown that the effectiveness of sgRNAs at targeting is variable, and so we recommend that multiple guides be tested until more robust design rules have been determined.

Next, to test multiplexed knockouts, we assembled guides in tandem on a single plasmid with Cas9, targeting one, two, three, or all four loci at once. The standardization and modularity of our assembly scheme made the construction of these multitargeting plasmids straightforward. We transformed these plasmids, again, with or without their cognate repair DNAs, and selected on synthetic media lacking uracil. We picked 40 colonies into different dropout media to determine the fraction of transformants that had the correct phenotype (auxotrophy). Consistent with results from Ryan *et al.*,<sup>51</sup> as the number of simultaneous targets increased, the fraction of correct transformants decreased, but it was still possible to disrupt all four targets at once with ~20% efficiency (Figure 9C). One possible cause of this decrease in efficiency could be recombination within the Cas9 plasmid that excises one or more guides, which could be minimized by using different promoters for each guide, but this has not been tested. Although the intention here is loss-of-function disruptions, only assaying for phenotype does not demonstrate the rate of correct repair DNA incorporation, as a nonhomologous end joining could also result in a disruption. To assay this, we screened six (three from each replicate) phenotypically correct colonies for each transformation by colony PCR. For the single, double, and



**Figure 9.** Multiplex, markerless knockouts. A plasmid expressing Cas9 and an sgRNA that targets the *LEU2* locus was transformed with (A) or without (B) a repair DNA that introduces a stop codon and destroys the sgRNA target. Shown are transformations plated on synthetic media lacking uracil, which selects only for the Cas9/sgRNA plasmid. Thus, selecting for the Cas9/sgRNA plasmid indirectly selects for cells that repaired the locus. (C) Multiple loci were targeted simultaneously, and 40 colonies each from two independent experiments were screened for the appropriate phenotype (auxotrophy). The raw number of colonies with and without repair is also shown to demonstrate that both the number of transformants and the fraction of correct clones decrease with an increasing number of targets. Finally, three colonies with the correct phenotype were then screened by colony PCR to verify proper integration of the repair DNA.

triple knockouts, all six colonies were correct, and for the quadruple knockout, four out of six were correct.

In addition to disrupting genes, this same strategy could be used to integrate large constructs in a markerless fashion or to introduce single nucleotide polymorphisms (SNPs). In cases where SNPs do not completely disrupt sgRNA targeting, two rounds of editing can be performed. The first round sgRNA should target the endogenous sequence, and the repair DNA should destroy the target and/or PAM by introducing a temporary, orthogonal sequence. The second round sgRNA should target the newly introduced sequence, and the repair DNA should reintroduce the endogenous sequence with the desired SNP. Although the same could be accomplished using a counterselectable marker such as *URA3*, using CRISPRm allows for multiple modifications to be made at once.

**Summary.** We have described a methodology and an accompanying toolkit of essential parts for engineering yeast. This MoClo-derived assembly standard supports the rapid cloning of multigene expression devices. We characterized a set of promoters and terminators, which are by no means exhaustive or perfect, but nonetheless diverse, in order to support the construction of multigene plasmids with minimal risk of unwanted homologous recombination. As a distinct method of controlling protein concentration, degradation tags are also characterized. Additionally, we have illustrated an important difference between using plasmids and chromosomal integrations and encourage expression from the chromosome whenever possible. To facilitate this, our system is designed to



make integrations as straightforward as plasmid transformations. We also present two options, using I-SceI or CRISPR/Cas9, for generating double-stranded breaks in the chromosome that increase integration efficiencies to match or even exceed that of plasmid transformations. Finally, we adapted the CRISPRm method to our standardized assembly scheme to enable multiplexed knockouts of endogenous genes. In summary, we believe this work will be a useful resource for both novice and experienced yeast biologists and engineers, and lays the foundation for a community that shares novel parts, as well as leads to greater consistency and reproducibility.

## METHODS

**Strains and Growth Media.** The *S. cerevisiae* strain used for measuring most promoters, terminators, degradation tags, copy number, and chromosomal integrations was BY4741 (*MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0*). The mating-type-specific promoters were also tested in BY4742 (*MATα his3Δ1 leu2Δ0 lys2Δ0 ura3Δ0*) and BY4743 (diploid cross of BY4741 and 4742). The galactose-induction experiments were conducted in a *GAL2* knockout of BY4741. The multiplex CRISPR/Cas9 knockout experiments were conducted in an S288C *MATa* haploid with a complete *URA3* coding sequence deletion.

Constitutive promoter and terminator characterization experiments were conducted in synthetic media with 2% (w/v) Dextrose (Fisher Scientific), 0.67% (w/v) Yeast Nitrogen Base without amino acids (VWR International), 0.2% (w/v) Drop-out Mix Complete w/o Yeast Nitrogen Base (US Biological), 0.85% (w/v) MOPS Free Acid (Sigma), 0.1 M Dipotassium phosphate (Sigma), 100 μg/L Zeocin (Life Technologies), buffered to pH 7.

Galactose inductions were performed in synthetic media with 2% (w/v) Raffinose (Fisher Scientific), 0.67% (w/v) Yeast Nitrogen Base without amino acids (VWR International), 0.2% (w/v) Drop-out Mix Synthetic Minus Uracil w/o Yeast Nitrogen Base (US Biological), plus 0–5% (w/v) Galactose (Fisher Scientific).

Copper inductions were performed in synthetic media with 2% (w/v) Dextrose (Fisher Scientific), 0.67% (w/v) Yeast Nitrogen Base without amino acids (VWR International), 0.2% (w/v) Drop-out Mix Complete w/o Yeast Nitrogen Base (US Biological), plus 0–50 μM Copper(II) Sulfate added.

All other experiments were conducted in synthetic media with 2% (w/v) Dextrose (Fisher Scientific), 0.67% (w/v) Yeast Nitrogen Base without amino acids (VWR International), 0.2% (w/v) Drop-out Mix Synthetic Minus appropriate amino acids w/o Yeast Nitrogen Base (US Biological).

YPD was used for preparing cells for transformation and recovery after heat shock: 1% (w/v) Bacto Yeast Extract, 2% (w/v) Bacto Peptone, 2% (w/v) Dextrose.

TG1 chemically competent *E. coli* was used for all cloning experiments. Transformed cells were selected on Lysogeny Broth (LB) with the appropriate antibiotics (ampicillin, chloramphenicol, or kanamycin).

**Yeast Transformations.** Yeast colonies were grown to saturation overnight in YPD, then diluted 1:100 in 50 mL of fresh media and grown for 4–6 h to OD<sub>600</sub>–0.8. Cells were pelleted and washed once with water and twice with 100 mM Lithium Acetate (Sigma). Cells were then mixed by vortexing with 2.4 mL of 50% PEG-3350 (Fisher Scientific), 360 μL of 1 M Lithium Acetate, 250 μL of salmon sperm DNA (Sigma), and 500 μL of water. DNA was added to 100–350 μL of

transformation mixture and incubated at 42 °C for 25 min. When selecting for prototrophy, the transformation mixture was pelleted, resuspended in water, and plated directly onto solid agar plates. When selecting for drug resistance, the transformation mixture was pelleted, resuspended in YPD, incubated at 30 °C for 2 h with shaking, pelleted and washed with water, then plated onto solid agar plates.

Plasmids designed for chromosomal integration (*i.e.*, containing 5' and 3' genome homology regions without a yeast origin of replication) were digested with NotI for 10 min prior to transformation to stimulate homologous recombination. The entire digestion reaction (without DNA cleanup) was included in the transformation in place of plasmid DNA.

**Golden Gate Assembly Protocol.** A Golden Gate reaction mixture was prepared as follows: 0.5 μL of each DNA insert or plasmid, 1 μL T4 DNA Ligase buffer (NEB), 0.5 μL T7 DNA Ligase (NEB), 0.5 μL restriction enzyme, and water to bring the final volume to 10 μL. The restriction enzymes used were either BsaI or BsmBI (both 10 000 U/mL from NEB). The amount of DNA inserts can optionally be normalized to equimolar concentrations (~20 fmol each) to improve assembly efficiencies.

Reaction mixtures were incubated in a thermocycler according to the following program: 25 cycles of digestion and ligation (42 °C for 2 min, 16 °C for 5 min) followed by a final digestion step (60 °C for 10 min), and a heat inactivation step (80 °C for 10 min). In some cases, where noted in the text, the final digestion and heat inactivation steps were omitted.

**Cloning of Parts.** See Supporting Information for details on construction of new parts.

**Promoter, Terminator, and Degradation Tag Characterization.** Promoter, terminator, and degradation tag testing constructs were integrated into the *URA3* locus of the yeast chromosome. Constitutive promoter, terminator, and degradation tag testing constructs were selected using a Zeocin resistance cassette; mating-type and inducible promoter testing constructs were selected for uracil prototrophy.

Colonies were picked and grown in 500 μL of media in 96-deep-well blocks at 30 °C in an ATR shaker, shaking at 750 rpm until saturated. Cultures were diluted 1:100 in fresh media, grown for 12–16 h, then diluted 1:3 in fresh media, and fluorescence was measured on a TECAN Safire2. For the galactose inductions, the media was switched during the dilution step from 2% dextrose to 2% raffinose with different concentrations of galactose. For the copper inductions, saturated cultures were diluted 1:100 in fresh media with different concentrations of copper(II) sulfate and grown for 18 h.

Excitation and emission wavelengths used to measure fluorescent proteins were mTurquoise2 at 435 nm/478 nm, Venus at 516 nm/530 nm, and mRuby2 at 559 nm/600 nm. Raw fluorescence values were first normalized to the OD<sub>600</sub> of the cultures, and then normalized to the background fluorescence of cells not expressing any fluorescent protein. The median log value of biological replicates was calculated and plotted with the range.

**Copy Number Characterization.** mRuby2 expression cassettes were assembled onto *URA3* plasmids or integrated into the *URA3* locus; Venus expression cassettes were assembled onto *LEU2* plasmids or integrated into the *LEU2* locus. For constructs where the two fluorescent proteins were expressed in tandem from the same locus/plasmid, they were assembled onto *URA3* plasmids or integrated into the *URA3*

locus; the strain used for these constructs was prototrophic for leucine.

Four colonies of each strain were picked into 400  $\mu\text{L}$  of synthetic media lacking uracil and leucine, and grown in 96-deep-well blocks at 30 °C in an ATR shaker, shaking at 750 rpm until saturated. The saturated cultures were measured for bulk fluorescence in a TECAN Safire2. The cultures were then diluted 1:100 into fresh media, grown for 4 h, and measured on a Fortessa X-20 flow cytometer.

Excitation and emission wavelengths used to measure bulk fluorescence on the TECAN were Venus at 516 nm/530 nm and mRuby2 at 560 nm/590 nm. Fluorescence values were normalized and reported in the same manner as the promoter characterization experiments.

The lasers and filters used on the flow cytometer were a 488 nm laser and a FITC filter (S05LP 530/30) for Venus; a 561 nm laser and PE-Texas Red filter (S95LP 610/20) for mRuby2. Voltages for each channel were kept constant for all samples at all copy numbers. Fluorescence was normalized for cell size by dividing by forward-scatter. Cytometry data were analyzed using FlowJo (<http://www.flowjo.com>).

Note: the selectable auxotrophic markers for uracil and leucine used in these experiments were different from those included in the toolkit. At the time these experiments were conducted, we had designed markers that encoded for the native Ura3p and Leu2p proteins, but used alternate codons for almost every position. We also used the respective terminator sequence from *Ashbya gossypii*, although we used the native *S. cerevisiae* promoter. The reason for these changes was an attempt to construct selectable markers with orthogonal sequences that would minimize undesired recombination with the chromosome, particularly for strains that did not have clean deletions of those genes as BY4741 does. Unfortunately, some of the changes resulted in a reduced growth rate on selective media, and were abandoned in favor of the native sequences. The only other experiment to use the alternative markers was the high-efficiency integration experiment (which also used *HIS3*).

**High-Efficiency Integrations.** The experimental strain used for the integration efficiency experiments was prepared by integrating the landing pad into BY4741 as depicted in Supporting Figure S4A. The repair DNA was constructed in two ways, with and without a CEN6/ARS4 origin. The plasmid with an origin was transformed and used to normalize the colony counts of all other transformations. The plasmid without an origin was linearized using NotI prior to transformation. The cutting plasmids expressing either I-SceI or Cas9/sgRNA were constructed onto CEN6/ARS4 plasmids with a *HIS3* selection marker, but were never selected for and were presumably present only transiently in cells. The cutting plasmids either were or were not also linearized with NotI prior to transformation. 100 fmol of each DNA (cutter and/or repair) was added to 350  $\mu\text{L}$  of transformation mix. After heat shock, 1/10th of the transformation was plated onto synthetic media lacking histidine. Pictures of the plates were taken and colonies were counted using Benchling (<https://benchling.com>).

**Multiplexed Knockouts.** 1  $\mu\text{g}$  of the Cas9/sgRNA plasmid ( $\sim 100$  ng/ $\mu\text{L}$ ) and 5  $\mu\text{g}$  of linear repair DNA ( $\sim 500$  ng/ $\mu\text{L}$ ) were added to 300  $\mu\text{L}$  of transformation mix. For the no repair controls, 10  $\mu\text{L}$  of water was added in place of the DNA. After heat shock, cells were washed with 300  $\mu\text{L}$  of water, pelleted,

and resuspended in 100  $\mu\text{L}$  of water and plated entirely onto synthetic media lacking uracil.

To screen for the knockout phenotype(s), 40 colonies were picked into 500  $\mu\text{L}$  of synthetic media lacking uracil in 96-deep-well blocks and grown at 30 °C in an ATR shaker, shaking at 750 rpm. Saturated cultures were washed twice in 500  $\mu\text{L}$  of water, then diluted 1:100 into four different media, each lacking the appropriate amino acid (leucine, histidine, methionine (and cysteine), or tryptophan). These cultures were then incubated again at 30 °C at 750 rpm, and we counted the number of clones that showed growth in the correct set of media.

Protospacer sequences for sgRNAs were designed using Benchling (see Supporting Table S2 for a list). See Supporting Figure S5A for details on the design of repair DNA.

## ■ ASSOCIATED CONTENT

### 📄 Supporting Information

All 96 plasmids described in this toolkit are available from Addgene (<http://www.addgene.org>). Supporting tables that list plasmids included in the toolkit, CRISPR/Cas9 protospacer, repair DNA sequences, and colony PCR primer sequences for verifying chromosomal integrations; supporting figures that provide additional data from experiments described in the text; supporting text and figures that describe and illustrate the assembly standard and methodology in greater detail; sequence files in GenBank format for the 96 plasmids included in the toolkit. This material is available free of charge *via* the Internet at <http://pubs.acs.org>.

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### Author Contributions

<sup>1</sup>M.E.L., W.C.D., and B.C. contributed equally to this work. M.E.L., W.C.D., and J.E.D. conceived the project. M.E.L., W.C.D., and B.C. performed all experiments and data analyses. M.E.L., W.C.D., B.C., and J.E.D. wrote the manuscript.

### Notes

The authors declare no competing financial interest.

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