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Puromycin and Methotrexate Resistance Cassettes and Optimized *cre*-recombinase Expression Plasmids for use in Yeast

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Abstract

Here we expand the set of tools for genetically manipulating *Saccharomyces cerevisiae*. We show that puromycin-resistance can be achieved in yeast through expression of a bacterial puromycin-resistance gene optimized to the yeast codon bias, which in turn serves as an easy to use dominant genetic marker suitable for gene disruption. We have constructed a similar DNA cassette expressing yeast codon-optimized mutant human dihydrofolate reductase (DHFR) that confers resistance to methotrexate and can also be used as a dominant selectable marker. Both of these drug-resistant marker cassettes are flanked by *loxP* sites allowing for their excision from the genome following expression of *cre*-recombinase. Finally, we have created a series of plasmids for low-level constitutive expression of *cre*-recombinase in yeast that allows for efficient excision of *loxP*-flanked markers.

Keywords

Saccharomyces cerevisiae; dominant markers; puromycin; methotrexate; *cre*-recombinase

Introduction

The budding yeast *Saccharomyces cerevisiae* has many intrinsic advantages as a model system to study a wide-range of biological processes. Beyond research, yeast is increasingly being used for synthetic production of proteins and compounds in industry. The *S. cerevisiae* genome is relatively small with few introns, and the completion of its sequencing (Goffeau, et al., 1996) has led to a range of tools for global operations and analysis. Examples include microarray analysis and use of genomic libraries, such as GFP-tag, TAP-tag, gene deletion and overexpression collections (DeRisi, et al., 1997; Ghaemmaghami, et al., 2003; Huh, et al., 2003; Winzeler, et al., 1999), these tools collectively provide a tremendous resource. As research targets larger protein complexes and networks, and industrial cell factory projects become more ambitious, demand increases for multiple gene modifications within a single experimental strain.

Conventional prototrophic markers are useful in yeast, but they rely on specific auxotrophic strains and can limit plasmid transformation options. These difficulties have been overcome in 2 main ways. One is using dominant selectable markers that confer drug-resistance, one example being the heterologous expression of the *E. coli* aminoglycoside 3' phosphotransferase (*kan^r*) that confers resistance to the aminoglycoside antibiotic

gentamicin / G418 (Wach, et al., 1994). Other dominant drug-resistance yeast markers that confer resistance to certain drugs have been described, for example nourseothricin and hygromycin B (Goldstein and McCusker, 1999), phelomycin (Gatignol, et al., 1987), cycloheximide (del Pozo, et al., 1991) and others. A second way to provide for multiple gene manipulations is using markers that can be subsequently excised and reused. For example, a marker flanked by tandem *hisG* repeats can accomplish this upon intragenic recombination (Alani, et al., 1987) or markers flanked by *loxP* sites can be excised from the genome using Cre-recombinase, which was first shown for a *loxP* containing *KanMX* cassette (Güldener, et al., 1996). Later, other markers, such as *URA3* from *Kluyveromyces lactis* and *his5⁺* from *Schizosaccharomyces pombe*, which complements *S. cerevisiae his3* mutants (Güldener, et al., 2002), were also developed. Despite various selectable markers, projects can still be constrained by consumption of such markers. In addition, some of the most effective drugs are not efficacious enough to eliminate a sequent laborious screening process to eliminate false positives. Finally, projects that require subsequent use of the same marker often use the Cre-*lox* system to regenerate markers. Commonly, Cre-recombinase production is controlled by the *GALI* promoter. However, many laboratory strains do not grow robustly on galactose, presenting a major problem with using *GALI*-driven Cre.

In an effort to expand the repertoire of genetic tools for yeast, we have generated two new dominant marker cassettes that can be used efficiently to delete a gene or register another genetic modification. These heterologous markers confer resistance to the drugs puromycin and methotrexate to wild-type parental strains of both haploid and diploid *S. cerevisiae*. Both cassettes are flanked by *loxP* sites, allowing easy marker excision using Cre-recombinase. Additionally, cassettes are housed in the backbone of a series of commonly used PCR template vectors (Güldener, et al., 2002), so that previously designed oligonucleotides are compatible with the new cassettes. Finally, we have optimized low-level constitutive expression of Cre-recombinase to allow fast marker retrieval, and created a series of selectable and counter-selectable *cre*-expression plasmids.

Materials and Methods

Reagents

A list of plasmids and yeast strains used and generated in this study are listed in Tables 1 and 2, respectively. Codon optimized versions of the bacterial *pac* gene and mutant human DHFR were designed for expression in *S. cerevisiae* and chemically synthesized by GenScript, Piscataway, NJ. The sequence data for the PCR template *puro^r* and *mtx^r* cassette vectors and Cre expression plasmids containing different nutritional markers have been deposited at NCBI GenBank and are available upon request from Addgene (The GenBank accession numbers and Addgene plasmid numbers are listed in Table 3).

Cell culture

Yeast Extract Peptone Dextrose (YPD) rich media (2% glucose, 2% peptone, 1% yeast extract) and synthetic complete (SC) minimal media (2% glucose, 1× yeast nitrogen base; Research Products International, Mount Prospect, IL), with appropriate amino acid and base drop out compositions for selections were used (Formedium, Norfolk, UK). Rich media

containing 1 mg/ml lead nitrate was prepared using a modified recipe (4% glucose, 0.3% peptone, 0.5% yeast extract, 0.02% ammonium sulfate). Geneticin (G418; Research products International, Mount Prospect, IL) was used at a concentration of 250 µg/ml in rich media. Puromycin (Gold Biotechnology, St. Louis, MO) was used at 4 mM for selection of strains carrying the *ptr5* mutation and 20 mM for wild-type background strains. Methotrexate (Sigma-Aldrich, St. Louis, MO) was used at a final concentration of 25 nM in synthetic complete plates. To minimize the expense of puromycin containing plates we routinely use 35 × 10 mm plates containing 2 mls of solidified agar media. Sulphanilamide (Fisher Scientific, Pittsburgh, PA) was added to methotrexate containing plates at a final concentration of 5 mg/ml. 5-fluoroorotic acid (5-FOA; GoldBiotechnology, St. Louis, MO) was added to SC plates at a final concentration of 1 mg/ml. 5-fluoroanthranilic acid (5-FAA; Matrix Scientific, Columbia, SC) was used in SC media at a concentration of 0.5 mg/ml.

Homologous recombination for gene deletion and plasmid construction

Yeast gene deletions were carried out by PCR based homologous recombination to integrate knockout cassette into desired locus. Oligonucleotides were designed containing ~20 nts of 3' sequence to prime from template DNA and ~50 nts of 5' sequence with homology to the region of integration. Most plasmids were also generated by homologous recombination, with amplified PCR products containing ~50 bp homology to the host vector, which was first linearized by restriction digest followed by gel extraction and purification (Qiagen, Valencia, CA). The generation of the *MET15* marked *TEF1**-cre plasmid (pPL5628) was carried out in the methionine auxotroph strain, BY4741. Plasmids generated by homologous recombination were rescued via SURE *Escherichia coli* cells (Stratagene, La Jolla, CA) and general DNA propagation was carried out in Top10 *Escherichia coli* cells (Invitrogen Life Technologies, Grand Island, NY). The final PCR template plasmids containing *puro^r* and *mtx^r* cassettes were created by standard sub-cloning methods, as detailed in Supplemental Figure S1.

Drug sensitive growth assays

Yeast cultures were grown overnight in SC media, or SC-URA media for cultures transformed with the *TEF1-pac* expression plasmid (pPL5103), diluted and grown for 6 hours to mid log phase. Cultures were then added to a sterile 96 well plate containing drugs as indicated. A 9-step, 10-fold serial dilution for each condition was created with a final volume of 250 µl in every well. Plates were incubated at 30°C overnight in a humidified incubator, mixed and then the average (n = 3) optical density at 600 nm (OD₆₀₀) was used to measure yeast growth. Graphs depict OD₆₀₀ compared with a control well lacking drug. These kill curves were used to estimate the lethal dose of either puromycin or methotrexate required for selection of cells expressing resistance markers. The sensitivity of yeast to certain drug concentrations was also confirmed on solid agar media.

Competitive fitness growth assays

Haploid yeast strains containing dominant drug marker integrations were grown to log phase for 6 hours in YPD media alongside control cell counterparts. Equal volumes of cultures

containing either the *puro^r* or *mtx^r* cassettes were then mixed with control cells and then diluted in a large volume of YPD media to allow continually growth at exponential phase. Cells were harvested at 1 hour, 24 hour and 48 hour time points, diluted 6000-fold and 9000-fold (pertaining to OD₆₀₀ = 0.5) before 200 µl of each sample was spread on a non-selective YPD plate and a drug selection plate. Plates were incubated at 30°C to allow growth prior to manual counting of colonies formed.

Immunoblot analysis

Yeast cells transformed with either pSH47 (*GALI-cre*) or pPL5071 (*TEF1*-cre*) were grown to mid-log phase in SC-URA media. Cultures expressing *cre* from the *GALI* promoter were grown in media containing 2% glucose, 2% raffinose or 2% galactose. Equivalent amounts of cells were harvested from each culture and incubated in 0.2 N NaOH for 3 minutes. Cells were then pelleted again and lysates generated by incubation in Laemmli sample buffer (50 mM Tris.HCl pH 6.8, 5% SDS, 10% glycerol) containing 8 M urea. Lysates were resolved by SDS-PAGE before transfer to nitrocellulose membrane for immunoblotting. Membranes were incubated in a blocking solution (5% milk) for 30 minutes prior to incubation with polyclonal anti-Cre-recombinase antibodies (catalogue #: PRB-106P-200; Covance Antibody Products, Dedham, MA). Immunoblot analysis of Carboxypeptidase Y was carried out as described (Macdonald, et al., 2012a) to serve as a loading control. Membranes were then probed with appropriate secondary antibodies conjugated to HRP. Protein levels were visualized by chemiluminescence and images were collected directly using a digital camera.

PCR analysis of genomic loci

Yeast cultures were grown to saturation in rich media before harvesting and 16 hours incubation in TE (50 mM Tris.HCl pH = 8.0, 20 mM EDTA, 1% 2-mercaptoethanol) buffer containing 200 µg zymolyase T-100. Genomic DNA was isolated by 2× phenol/chloroform/isoamyl (25:24:1) extractions followed by ethanol precipitation. DNA was resuspended in water and used as a template for PCR reactions.

Routine protocol for using new reagents

Puromycin (*puro^r*) and methotrexate (*mtx^r*) resistance cassettes were amplified by PCR using 50 ng of template plasmid. DNA was ethanol precipitated, resuspended in water and transformed into competent yeast using standard Lithium acetate methods wherein yeast were pelleted from LiOAc-Sorbitol-PEG suspension, resuspended in water, and plated directly onto the drug-containing plate (Gietz and Woods, 2002). Puromycin-resistant yeast from wild-type parents were selected for on small (2 ml) YPD plates containing 20 mM puromycin. Reduced drug concentrations are sufficient for selection of hypersensitive strains, discussed in Figure 3. Methotrexate-resistant yeast were selected on plates containing SC minimal media replete with all amino acids and containing 5 mg/ml sulphanilamide and 50 nM methotrexate. In practice, we added drugs after autoclaving media, however, we found no reduced efficacy of sulphanilamide if the drug is added prior to autoclave sterilization. Drug-resistant single colonies were then patched on fresh selection plates before yeast is grown for isolation of genomic DNA and genotyping. For excision of

loxP-flanked cassettes, yeast were transformed with the *TEF1**-*cre* plasmid. Yeast were selected on minimal media plates lacking the appropriate nutrient (e.g. SC-Ura for pPL5071). Single colonies were patched onto fresh plates before preparation of genomic DNA for genotyping.

Results & Discussion

Puromycin-resistance as a dominant marker in yeast

Puromycin is an antibiotic derived from *Streptomyces alboniger* that induces chain termination during protein translation by releasing the ribosome (Traut and Monro, 1964). Puromycin is inactivated by puromycin *N*-Acetyltransferase encoded by the *S. alboniger pac* gene (Lacalle, et al., 1989; Perez-Gonzalez, et al., 1985; Vara, et al., 1985). Exogenous expression of *pac* has been used as a dominant marker in mammalian cells (de la Luna, et al., 1988; Vara, et al., 1986), and more recently in *C. elegans* (Semple, et al., 2010). We titrated concentrations of puromycin in growth media cultures of *S. cerevisiae* to test whether puromycin could effectively kill wild-type yeast. We also tested cells lacking the multi-drug transporter Pdr5, which are hypersensitive to a wide range of drugs (Ernst, et al., 2005). We found that growth of wild-type yeast is completely inhibited in the presence of 20 mM puromycin, and that growth of *pdr5* cells was completely inhibited at 2.5 mM (Figure 1A). We took advantage of the hypersensitive *pdr5* strain, which also harbours a Kanamycin-resistance (*kan*^r) cassette at the *UBI4* locus, to test if puromycin-resistance was a viable selection in yeast. We chemically synthesized a version of the *pac* gene with an optimized codon sequence for expression in yeast and targeted its integration by homologous recombination between the *TEF1* promoter and terminator present in the *kan*^r cassette (Figure 1B). Puromycin-resistant (*puro*^r) colonies were apparent after 48 hours following transformations containing the *pac* gene PCR product, with no colonies in the control containing only carrier DNA (Figure 1C). Correct insertion of the *pac* gene at the *UBI4* locus corresponded with replacement of the *kan*^r cassette, demonstrated by failure of *puro*^r clones to grow in the presence of G418, unlike the parental strain (Figure 1D). Amplifying the *UBI4* locus by PCR was used to confirm the genotype of the *puro*^r strains (Figure 1E). We next determined how well the *puro*^r cassette functioned in wild-type cells by first introducing it on a low copy *CEN*-based plasmid (pPL5103). We found that *pac* gene expression was sufficient to confer resistance to 20, 30 and 40 mM puromycin, concentrations that prevent growth of wild-type cells (Figure 1F).

Methotrexate-resistance as a dominant marker in yeast

The Dihydrofolate reductase (DHFR) enzyme catalyses the NADPH-dependent conversion of dihydrofolate to tetrahydrofolate, an essential co-factor in metabolic processes. Methotrexate is a commonly used drug that inhibits DHFR, with subsequent loss of tetrahydrofolate that induces cell death; conversely, cells expressing high levels of DHFR are resistant to methotrexate (Biedler, et al., 1972; Chang and Littlefield, 1976; Littlefield, 1969). Methotrexate-resistance through increased expression of DHFR was developed as a dominant marker in mammalian cells (Christman, et al., 1982; Murray, et al., 1983; Wigler, et al., 1980). This concept was then adapted for use in yeast (Miyajima, et al., 1984). As the authors documented, methotrexate is inefficient at killing yeast cells unless sulfanilamide is

added, to block the *de novo* synthesis of dihydrofolate (Brendel, et al., 1975; Wickner, 1975). Similarly we found growth is completely inhibited in yeast grown in the presence of 20 nM methotrexate and 5 mg/ml sulphanilamide, but a limited level of growth is detected at all concentrations of methotrexate tested in cultures lacking sulphanilamide (Figure 2A). It is unclear why sulfanilamide, which blocks synthesis of the dihydrofolate precursor, dihydropterate, from the *de novo* pathway is required because DHFR and methotrexate function after these pathways converge, presumably some functionality of thymidine synthesis bypasses this canonical route. Regardless, the combined lethal effect of methotrexate and sulphanilamide provides a useful system to test exogenous expression of DHFR to overcome methotrexate-sensitivity. It was originally hypothesized that naturally occurring mutants of DHFR with decreased affinity for methotrexate were more resistant to the drug (Nakamura and Littlefield, 1972). Later biochemical studies were carried out using murine DHFR showing various mutants do decrease the binding affinity of DHFR to methotrexate (Thillet, et al., 1988). Combining two of these mutants, L22F and F31S, located in the methotrexate binding region of DHFR, (Figure 2B) results in a version of DHFR that is approximately 10,000 times less sensitive to methotrexate than endogenous yeast DHFR (Remy, et al., 2007). Because this was such an effective selection tool for carrying out protein interaction complementation assays (Tarassov, et al., 2008), we created a codon optimized double mutant DHFR gene for use as a methotrexate-resistant (*mtx^r*) dominant marker. A homologous recombination strategy was used to insert the gene between the transcriptional (*TEF1*) machinery of a *his5⁺* knockout cassette (Figure 2C). Recombination was carried out in a *bro1⁻ ::his5⁺* deletion strain (Pashkova, et al., 2013), and selected for on methotrexate plates containing sulphanilamide (Figure 2D). Methotrexate-resistant transformants were then unable to grow on media lacking histidine (-His), demonstrating that the mutant *DHFR* gene had successfully recombined at the *BRO1* locus and conferred resistance to methotrexate, this was also confirmed by PCR analysis of the *BRO1* locus (Figure 2E & F).

New loxP flanked marker cassettes

Having shown that expression of the *pac* gene and mutant DHFR in yeast confers resistance to puromycin and methotrexate, respectively, we next cloned the dominant marker cassettes into vectors for use as PCR templates. Upon sequencing, the *mtx^r* cassette rescued from methotrexate-resistant yeast was found to contain an additional mutation (F180L), besides the two intentional L22F and F31S amino acid changes that reduce DHFR affinity for methotrexate. The conservative F180L substitution is housed in a β -sheet domain distinct from the methotrexate-binding domain: a hydrophobic pocket facing the opposite direction (Figure 2B). Based on the location of the F180L mutation it is unlikely to impact resistance to methotrexate, although we cannot exclude the possibility it enhanced survival chances of the original methotrexate-resistant yeast. Because this version of mutant DHFR was very effective at its intended purpose (see below), no further modification of it was undertaken.

We chose a vector backbone commonly used for generation of dominant marker cassettes, plasmid pUG27, to host the puromycin and methotrexate-resistant markers (Supplemental Figure S1). pUG27 encodes a *his5⁺* marker flanked by *loxP* sites (Gueldener, et al., 2002), so we simply inserted the *puro^r* and *mtx^r* genes in place of *his5⁺*, retaining the same

promoter and terminator sequences and the *loxP* sites for recombination based excision (see below). Another advantage to this strategy is that the *puro^r* and *mtx^r* cassettes can be amplified using oligonucleotides designed for use with existing pUG vectors. To generate cassettes, oligonucleotides that anneal to the common vector sequences should be designed contiguous with 40-50 nts of 5' DNA sequence homologous to the region of integration that is being targeted.

In order to test these cassettes, we next generated a *puro^r* PCR product to integrate in place of the *ADE2* gene of *S. cerevisiae*. Resistant clones were used to compare the drug-sensitivity of yeast on solid agar. We used the kill curves derived from liquid cultures (Figure 1) as an estimate of drug concentrations required and found the selectivity to be similar. We found that 8 mM puromycin was a sub-lethal dose but had a marked effect on the growth of both haploid and diploid wild-type cells; increasing the concentration of puromycin to 20 mM was sufficient to completely inhibit growth (Figure 3A). By contrast, both diploid and haploid yeast containing the *puro^r* cassette could grow normally at 20 mM puromycin.

It has recently been proposed that wild-type yeast cells are insensitive to puromycin, and that a combination of gene deletions (*pdr1*, *pdr3* and *erg6*) are required to sensitize cells to the drug (Cary, et al., 2014). In agreement with this study we find perturbations in the *ERG* (ergosterol biosynthesis) and *PDR* (pleiotrophic drug response) pathways do sensitize cells to puromycin. The most effective single gene deletion to sensitize cells to puromycin was *PDR5*. As mentioned in Figure 1, the *ubi4 pdr5* strain (originally used to integrate the *pac* gene) cannot grow in puromycin concentrations of ~2.5 mM. This is supported by observations on solid agar where 4 mM puromycin is sufficient to kill *ubi4 pdr5* cells, and 2 mM puromycin inhibits growth significantly (Figure 3B). We attribute this drug sensitivity to the absence of Pdr5, because single *pdr5* mutant cells exhibit the same sensitivity. Deletion of *PDR1* or *ERG6* did sensitize cells to puromycin but to a lesser extent. Taking into account the inherent slow growth defect observed in *erg6* null cells (Gaber, et al., 1989; Welihinda, et al., 1994), *erg6* and *pdr3* cells have very similar puromycin-sensitivity, where most growth was inhibited at 8 mM. The growth of *pdr3* cells in the presence of puromycin was indistinguishable from wild-type, as shown previously for other drugs (Prunuske, et al., 2012); it may be the puromycin-sensitivity imparted through this mutation is too subtle for detection at the concentrations tested. Our finding that *PDR5* deletion increases drug sensitivity more than cells lacking either of the Pdr5 upstream transcriptional regulators, Pdr1 or Pdr3, is consistent with previous findings (Dexter, et al., 1994; Meyers, et al., 1992). Although deletion of such genes allows use of lower concentrations of puromycin - for *puro^r* selection or study of translation inhibition - the myriad defective phenotypes associated with each deletion might undermine the physiological relevance to such experiments. We would recommend simply using the higher doses of puromycin documented herein, an approach that is not obliged to any particular genotype.

We also tested methotrexate-sensitivity of haploid and diploid cells by integrating a PCR product encoding the *mtx^r* cassette at the *MET15* locus. We find the sub-lethal concentration of 10 nM methotrexate dramatically slows growth of haploid and diploid wild-type yeast

and concentrations above 20 nM is sufficient to effectively kill both strains (Figure 3C). We routinely use 50 nM methotrexate for selection, a concentration at which both haploid and diploid yeast carrying an integrated *mtx^r* cassette grow efficiently.

We find the puromycin and methotrexate concentrations required to affect lethality in yeast were remarkably similar in liquid and solid media. The concentrations we document should provide useful estimates for drug titrations to determine the sensitivity of other experimental strains. We also find the ploidy state does not alter the required drug concentrations to select for yeast expressing the respective resistance cassettes. Finally, we carried out competitive fitness growth assays of yeast integrated with either the *puro^r* or *mtx^r* cassettes and find them to be phenotypically neutral (Supplemental Figure S2), as has been shown for other dominant drug resistance markers (Goldstein and McCusker, 1999; Vorachek-Warren and McCusker, 2004). Together, these features expand the potential use of these dominant markers.

Constitutive Cre-recombinase expression plasmids for marker retrieval

The ability to remove integrated markers allows further use of that selection tool in subsequent rounds of genetic manipulation. A widely used system for this is based on the site-specific recombination of short DNA sequences, called *loxP* sites, by Cre-recombinase in phage P1 (Abremski, et al., 1983; Hoess and Abremski, 1985; Sternberg and Hamilton, 1981). Expression of *cre* in yeast to excise a *loxP* flanked *LEU2* marker to return cells to a *Leu⁻* phenotype was the first documentation of this system in eukaryotes (Sauer, 1987). This study induced expression of *cre* from the *GAL1* promoter, which was useful in demonstrating specificity of Cre activity, and has since been used to remove other integrated *loxP* containing dominant marker cassettes (Guldener, et al., 2002). This approach involves shifting the carbon energy source to galactose in order to induce *cre* expression, a significant physiological change highlighted by a slow growth phenotype (Johnston, 1987; Lohr, et al., 1995). Indeed, certain commonly used strains grow very poorly on galactose (Supplemental Figure S2). To bypass extended incubation times we designed a plasmid that constitutively expresses *cre*-recombinase from a modified *TEF1* promoter of moderate strength (*TEF1**). The *TEF1** promoter shares 95% sequence identity with the wild-type version but sustains only a 16% level of expression (Nevoigt, et al., 2006). We exchanged the *GAL1* promoter of plasmid pSH47 (Guldener, et al., 1996) with that of the *TEF1** by homologous recombination (Figure 4A), as confirmed by PCR analysis (Figure 4B) and sequencing.

We next measured the levels of Cre by immunoblot analysis of yeast lysates generated from cells transformed with either the *GAL1* or *TEF1** driven *cre* plasmids. As expected, the *GAL1* promoter produced high levels of Cre in cells when grown in media containing galactose, and there were no detectable levels when cells were grown in media containing glucose (repressed state) or raffinose (Figure 4C). The constitutive expression of *cre* from the *TEF1** promoter was markedly less than *GAL1* induced levels. Immunoblots from multiple experiments were quantified by densitometry and show Cre levels expressed from the *TEF1** promoter are ~12% of that from the *GAL1* promoter induced with galactose (Figure 4D). We next compared the how efficient each plasmid was at mediating Cre-induced recombination between *loxP* sites in cells containing a *loxP-his5⁺-loxP* marker

cassette at the *BRO1* locus. We found cells expressing the *TEF1*-cre* plasmid formed colonies after 48 hours and 100% of transformants tested had correctly excised the *loxP*-flanked marker cassette, as indicated by histidine auxotrophy. Cells containing *GALI-cre* also formed colonies on glucose plates in 48 hours, and none of these had excised the *loxP-his5⁺-loxP* marker, consistent with glucose repression of Cre production. When these cells were patched onto solid agar containing galactose they efficiently excised the *his5⁺* marker, but patches took an additional 48 hours to grow. An alternative strategy to avoid extended incubations when using the *GALI-cre* plasmid is to expose them to galactose for “pulse” periods, however, we found that even an 8 hour galactose pulse only resulted in ~28% of cells successfully excising the *loxP* flanked *his5⁺* marker (Figure 4E).

The *TEF1*-cre* plasmid achieves maximal excision efficiency in much less time than the *GALI-cre* plasmid, and does not require additional manipulations to shift cells to another carbon source. It has been shown in yeast containing multiple *loxP* sites, promiscuous Cre activity can force undesired recombination events, sometimes inducing severe chromosomal rearrangements (Delneri, et al., 2000). To ensure that low-level constitutive expression of *cre* in cells harbouring the *TEF1*-cre* plasmid did not significantly increase the likelihood of such promiscuous activity, we generated a yeast strain containing three *loxP* flanked markers. First, the nutritional marker genes *ADE2* and *MET15* were deleted using the *puro^r* and *mtx^r* marker cassettes, respectively. Then a *his5⁺* cassette was used to delete *SNA3*, which encodes a small membrane protein involved with endocytic trafficking (MacDonald, et al., 2012b) (Figure 4F). We then excised the markers from the triple null strain using either the *TEF1*-cre* or *GALI-cre* expression plasmids. Genomic DNA was isolated from 24 single colony transformants from each test group and PCR analysis was used to confirm marker excisions at each locus. We found that all 72 *loxP*-marked loci were correctly excised when Cre was generated using the *TEF1** plasmids (Figure 4F and Supplemental Figure S3). We found similar success rates with the *GALI* plasmid, although it took much longer to obtain transformants lacking the three markers. From these studies we conclude that *TEF1*-cre* is no worse at inducing promiscuous Cre activity than the *GALI-cre* version, and it is possible that the approximately 10-fold reduced Cre levels in cells carrying the *TEF1*-cre* plasmid actually minimize these unwanted effects. Promiscuous Cre activity observed in our experimental conditions were less than documented by Delneri et al, who found ~75% success from a strain housing 4 *loxP* marked cassettes. Presumably the total number of *loxP* sites in a strain, the particular marker cassettes integrated, and the genomic localization all contribute to the likelihood of undesired recombination events. It is also possible that some chromosomal rearrangements prove lethal and are selected against during *cre* expression. We caution that proper genotyping of any modified loci should be carried out following genetic manipulation to ensure the desired modification has been achieved.

The *TEF1*-cre* vector discussed above (pPL5071) is a *CEN* based low copy expression plasmid containing a *URA3* selection marker (Figure 4F). We created additional versions of this *cre* expression plasmid carrying different selection markers: *ADE2* (pPL5606), *TRP1* (pPL5608) and *MET15* (pPL5627). *URA3*, *TRP1*, and *MET15* versions can be removed by counter-selection, additionally, the *ADE2* and *MET15* versions can be removed by growth in

non-selective media, and identified by a simple change in colony colour upon plasmid loss (Figure 4G; discussed in more detail below).

Use of new tools for gene deletion and marker retrieval

To document the utility of the reagents discussed in this manuscript we used them to create additional auxotroph versions of the commonly used lab strain BY4742 (Brachmann, et al., 1998). Firstly, a puromycin-resistance cassette was integrated at the *ADE2* locus and *puro^r* colonies were tested for growth on puromycin and SC-Ade plates (Figure 5A). The resultant *ade2 ::puro^r* strain was transformed with our *URA3* marked *TEF*-cre* plasmid, in order to remove the *puro^r* cassette and create an *ade2 ::loxP* strain. The *URA3*-containing *cre* plasmid was then removed by selecting cells on SC plates containing 5-fluoroorotic acid (5-FOA; Figure 5A, right), a counter selection tool that only allows growth of cells that have lost the *URA3*-containing plasmid (Boeke, et al., 1984). *URA3*-containing Cre plasmids cannot be used in *Ura⁺* strains, which presents a problem for strains in which a *loxP-URA3-loxP* cassette had been deployed (Gueldener, et al., 2002). Similar to before, we deleted the endogenous copy of *ADE2* using a *loxP-URA3-loxP* marker and also made an *ADE2* version of *TEF1*-cre*, which efficiently excised the *URA3* marker (Figure 5B). Although there is no known counter selection for *ADE2* to select for cells lacking the *ADE2-TEF1*-cre* plasmid, there is an easy counterscreen. Yeast that had successfully lost the *ADE2-cre* plasmid were easily identifiable by their red colony colour phenotype (Figure 5B), due to the accumulation of phosphoribosylaminoimidazole, the Ade2 substrate that generates a red polymer following oxidization in *ade2* auxotrophs (Smirnov, et al., 1967). This tool could be used to confirm loss of *TEF1*-cre* in any *ade2* mutant yeast, such as the commonly used W303 (Rothstein, 1983) and YNN/YPH (Sikorski and Hieter, 1989) strains.

We next used the *mtx^r* cassette to delete the *MET15* gene, which causes a methionine auxotroph phenotype (Massetot and De Robichon-Szulmajster, 1975). We transformed a version of *TEF1*-cre* plasmid containing the *MET15* nutritional marker into *met15 ::mtx^r* cells to excise the marker (Figure 5C). There are two options available to removal of the *MET15-TEF1*-cre* plasmid. First, there was counter selection for cells resistant to methylmercury, which selects against *MET15* expressing yeast as documented previously (Singh and Sherman, 1974). Second, was a blackening of colony colour when *met15* auxotrophs are grown on modified rich media containing Pb^{2+} ions (Cost and Boeke, 1996). This allows visual identification of colonies that have lost the *cre* plasmid and was found to be easily implemented (Figure 5C) thus avoiding use of hazardous methylmercury. Finally, we created a version of the *TEF1*-cre* plasmid carrying the *TRP1* marker. Figure 5D shows that *trp1 ::his5⁺* cells could be cured of their *TRP1-TEF1*-cre* plasmid following growth on plates with 5-fluoroanthranilic acid (5-FAA), which selects against cells expressing *TRP1* (Toyn, et al., 2000).

Summary

In this paper we document that there are sufficient levels of puromycin that can effectively inhibit yeast growth and be used to select for yeast that express a puromycin-resistance gene. This is a useful addition to the list of options for dominant marker selection in yeast. It was previously documented that three deletions (*pdr1* , *pdr3* and *erg6*) were necessary to

elevate intracellular drug levels to allow puromycin-based inhibition of translation (Cary, et al., 2014). It is useful to document that higher doses of puromycin can also achieve this goal, if the desired experiments were to be carried out in a strain other than *pdrl*, *pdrl3* *erg6*, a strain that is not ideal for many experimental questions. We also show that methotrexate-resistance in yeast can be used as convenient dominant selection tool. The cloning of the *puro^r* and *mtx^r* markers into the vector backbone of the pUG PCR template vector series allows easy incorporation of these tools with existing methods. Finally, we document the use of plasmids that constitutively express *cre*-recombinase that allows for quick excision of chromosomal regions flanked by *loxP* sites. This series of plasmids containing different prototrophic markers, all of which can be removed easily, allows use in a variety of yeast strain backgrounds.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Puromycin-resistance as a selective marker in yeast

A) Wild-type and *pdr5* cells were grown to mid-log phase in SC media before addition to a 2-fold serial dilution of puromycin in a 96-well plate. Each well contained a 250 μ l culture of yeast cells at an $OD_{600} = \sim 0.005$. The puromycin gradient ranged from 40 mM to 0.04 mM, with a final well containing no drug. Cells were incubated at 30°C for 72 hours, mixed, and then the OD_{600} was measured. Growth of wild-type (blue) and mutant *pdr5* (green) cells is indicated.

B) Schematic diagram of homologous recombination strategy to replace the Kanamycin-resistance (*kan^r*) cassette at the *UBI4* locus with that of the *pac* gene conferring Puromycin-resistance (*puro^r*), retaining the original *TEF1* promoter and 3' untranslated (UTR) terminator sequences for expression of the new marker.

C) *ubi4^Δ::kan^r* cells (also containing *pdr5* mutation) were transformed with either a PCR product encoding the *pac* gene or a carrier DNA control. Cells were plated on YPD plates containing 4 mM puromycin and colonies were imaged after 48 hours.

D) Puromycin-resistant clones from (C) were patched onto plates containing puromycin or G418. As a control the parental *kan^r* is included.

E) PCR analysis of the *UBI4* locus from genomic DNA isolated from wild-type yeast or yeast containing either the *kan^r* or *puro^r* cassettes at the *UBI4* locus.

F) Growth assay as described in (A) of wild-type cells expressing *TEF1-pac* from a low-copy *CEN* plasmid (pPL5103) (red). The sensitivity of wild-type cells to puromycin from (A) is indicated (dotted blue).

Figure 2. Mutant DHFR confers methotrexate-resistance in yeast

A) Wild-type cells were grown to mid-log phase before addition to the wells of a 96-well plate containing a 2-fold serial dilution of methotrexate. After 72 hours incubation at 30°C growth measured by absorbance at 600 nm was calculated as a percentage of yeast growth in media lacking drug. Growth across the methotrexate gradient is indicated (red), as is growth in the same gradient containing 5 mg/ml sulphanilamide (blue).

B) The crystal structure of human DHFR in complex with the methotrexate ligand, (PDB accession number 1U72) is depicted. hDHFR is shown in grey cartoon format, the two mutations that reduce affinity to methotrexate (L22F and F31S) are shown in cyan and an arrow indicates the methotrexate-binding pocket. The location of an additional mutation (F180L) acquired in the selection for methotrexate-resistant yeast clones is shown in magenta.

C) Schematic diagram of homologous recombination strategy to replace the *his5⁺* cassette between the *TEF1* machinery at the *BRO1* locus with that of the mutant form of human DHFR carrying the L22R and F31S mutations (*DHFR** / *mtx^r*).

D) *bro1 ::his5⁺* cells were transformed with either a PCR product encoding *DHFR** or carrier DNA control. Cells were plated on SC plates containing 20 nM methotrexate and 5 mg/ml sulphanilamide.

E) Methotrexate-resistant clones from (D) and the parental *bro1 ::his5⁺* strain were patched onto plates containing methotrexate and sulphanilamide and plates lacking histidine (-His).

F) Genomic DNA isolated from wild-type, *bro1 ::his5⁺*, and *bro1 ::mtx^r* yeast were analysed by PCR using oligonucleotides in the 5' and 3' untranslated regions of the *BRO1* locus.

Figure 3. Puromycin and methotrexate selections on solid agar media

A) Haploid and diploid cells, both wild-type parents and strains carrying a *puro^r* cassette, were grown to mid-log phase before dilution and plating. Cultures were plated on rich media containing either 8 mM or 20 mM puromycin, and a control YPD plate that lacked drug.

B) Wild-type cells or strains carrying gene deletions indicated were grown to mid-log phase before plating on YPD plates containing different concentrations of puromycin.

C) Haploid and diploid cells, either wild-type or containing a *mtx^r* cassette were grown in liquid culture, diluted and then plated on SC media. Cultures were also plated on SC media containing 5 mg/ml sulphanilamide and either 10 nM or 80 nM methotrexate.

Figure 4. Expression of cre-recombinase from a constitutive promoter

A) Schematic diagram depicting the homologous recombination strategy used to create *TEF1*-cre* plasmid (pPL5071) using the *GAL1-cre* parent plasmid, pSH47 (Güldener, et al., 1996).

B) Confirmation of the replacement of *GAL1* promoter with that of *TEF1** by PCR using oligonucleotides that anneal in common regions either side of the promoter sequences. The slightly smaller PCR product generated from the *TEF1** template migrates faster. *HpaI* digestion, a restriction site unique to the *TEF1** promoter, was used to confirm the *GAL1* promoter has been exchanged.

C) Yeast transformed with the *GAL1-cre* plasmid were grown to mid-log phase in SC-Ura media supplemented with either galactose (Gal), raffinose (Raf) or glucose (Glu). Yeast retaining the *TEF1*-cre* plasmid were also grown in glucose containing SC-Ura media. Cells were harvested and treated with 0.2 N NaOH for 3 minutes before lysates were

generated by addition of Laemmli sample buffer containing 8 M urea. Lysates were resolved by SDS-PAGE followed by transfer to nitrocellulose and probing with polyclonal antibodies raised against Cre and CPY.

D) Quantitation of Cre levels driven from either the *GAL1* promoter in different carbon sources or the *TEF1** promoter. Densitometry of Cre immunoblots was carried out using Fiji image analysis software and the mean and SD shown from 3 experiments are shown.

E) *bro1* Δ *loxP-his5⁺-loxP* cells were transformed with *TEF1**-cre (white) or *GAL1*-cre (black) plasmids were selected for on glucose containing SC-Ura plates. Single colony transformants (n = 48 each) were patched on SC-His plates and the percentage of cells that had successfully excised the *loxP-his5⁺-loxP* marker are shown. Transformants were also washed 3x in SC-Ura galactose media and then grown for 1 hour or 8 hours in galactose media before being washed 3 times in SC-Ura glucose media to repress *GAL1* induction of Cre. Cultures were struck out on SC-Ura glucose plates and the percentage of single colony transformants that had excised the *his5⁺* marker were identified by patching on SC-His plates.

F) The triple null *ade2 met15 sna3* strain containing *loxP* flanked *puro^r*, *mtx^r* and *his5⁺* markers, respectively, was grown on YPD plates, YPD 20 mM puromycin, SC + 50 nM methotrexate, or SC-His plates. As a control the parental wild-type strain By4742 was included. The excision of *loxP* markers from *ade2 met15 sna3* cells transformed with either *TEF1**-cre (white) or *GAL1*-cre (black) is shown. Cre efficiency was calculated by PCR analysis of the *ADE2*, *MET15* and *SNA3* loci from genomic DNA isolated from single colony transformants of each plasmid (24 each), shown in supplemental Figure S3.

G) Plasmid map of pPL5071 encoding *TEF1**-cre from a low-copy *URA3* marked *CEN* plasmid.

H) Table of *TEF1**-cre plasmids including the size of each vector, the positive selection marker, and the means to remove plasmid.

Figure 5. Use of new dominant markers and TEF1*-cre plasmids

A) The *ADE2* gene of wild-type yeast (BY4742) was deleted using the *loxP-puro^r-loxP* cassette. The *puro^r* cassette was removed using pL5071, a *URA3* marked plasmid expressing *cre*-recombinase from a mutant *TEF1** promoter. pL5071 was removed from *ade2::loxP* cells by growth on 5-FOA, single colonies were selected and plated on 5-FOA and -Ura media to confirm plasmid loss.

B) As with (A) the *ADE2* gene of BY4742 was deleted, this time using a *URA3* deletion cassette generated from PCR template pUG72 (Guedener, et al., 2002). The *URA3* marker was excised by *cre* expression from the *ADE2* marked version (pL5606), which was subsequently removed by growth on rich media and screening for red single colonies. Cells were plated on SC minimal media and incubated for an extra 24 hours to develop red colour for clarity.

C) The *MET15* gene of BY4742 was deleted using the *loxP-mtx^r-loxP* dominant marker cassette. The *mtx^r* cassette was removed by expression of *cre*-recombinase from pL5627, which carries a *MET15* marker. This plasmid was removed by growth on rich media, and cells lacking the plasmid could be easily identified on modified YPD media containing Pb²⁺

ions. Cells were incubated for an extra 48 hours at 4°C to develop colour difference for documentation in this image.

D) The *TRP1* locus of BY4742 was deleted using a *his5⁺* cassette amplified from pUG27 (Gueldener, et al., 2002). The marker was removed using the *TRP1* marked *cre* expression plasmid (pPL5608), which was later removed by selective growth on SC media containing 5-FAA.

Table 1
Yeast strains used in this study

Strain	Genotype	Source
BY4741	MATa <i>his3 leu2 met15 ura3</i>	(Brachmann et al., 1998)
BY4742	MATa <i>his3 leu2 lys2 ura3</i>	(Brachmann et al., 1998)
SEY6210	MATa <i>leu2-3,112 ura3-52 his3- 200 trp1- 901 lys2-801 suc2- 9</i>	(Robinson et al., 1988)
EE10	MATa SEY6210	From Scott Moye-Rowley
X2180-1A	MATa <i>SUC2 mal mel gal2</i>	(Mortimer and Johnston, 1986)
X2180-1B	MATa <i>SUC2 mal mel gal2</i>	(Mortimer and Johnston, 1986)
PLY4008	BY4742; <i>pdv5 :: his5⁺ ubi4 ::kan^r</i>	This study
PLY4541	BY4742; <i>pdv5 :: his5⁺ ubi4 ::puro^r</i>	This study
PLY4225	SEY6210; <i>bro1 ::his5⁺</i>	(Pashkova et al., 2013)
PLY4650	SEY6210; <i>bro1 ::mtx^r</i>	This study
PLY4689	BY4742; <i>ade2 ::puro^r</i>	This study
PLY4752	Diploid; BY4741 × BY4742	This study
PLY4792	Diploid; BY4741 × BY4742 <i>ade2 ::puro^r</i>	This study
PLY4774	BY4742; <i>pdv5 ::kan^r</i>	(Winzeler et al., 1999)
PLY4772	BY4742; <i>pdv1 ::kan^r</i>	(Winzeler et al., 1999)
PLY4773	BY4742; <i>pdv3 ::kan^r</i>	(Winzeler et al., 1999)
PLY4775	BY4742; <i>erg6 ::kan^r</i>	(Winzeler et al., 1999)
PLY4757	BY4742; <i>ade2 ::puro^r ; met15 ::mtx^r</i>	This study
PLY4760	Diploid; BY4741 × BY4742 <i>ade2 ::mtx^r</i>	This study
PLY4778	BY4742; <i>ade2 ::puro^r met15 ::mtx^r sna3 ::his5⁺</i>	This study
PLY4797	BY4742; <i>ade2 ::loxP met15 ::loxP sna3 ::loxP</i>	This study
PLY4721	BY4742; <i>ade2 ::loxP</i>	This study
PLY4682	BY4742; <i>ade2 ::URA3</i>	This study
PLY4641	BY4742; <i>trp1 ::his5⁺</i>	This study
PLY4721	BY4742; <i>trp1 ::loxP</i>	This study
PLY4678	BY4742; <i>met15 ::mtx^r</i>	This study
PLY4719	BY4742; <i>met15 ::loxP</i>	This study

Table 2

Plasmids used in this study.

Plasmid	Description	Source
pRS416	Low copy yeast shuttle plasmid containing <i>URA3</i> marker	(Sikorski and Hieter, 1989)
pRS414	Low copy yeast shuttle plasmid containing <i>TRP1</i> marker	(Sikorski and Hieter, 1989)
pPL5103	pRS416 expressing <i>pac</i> from <i>TEF1</i> * promoter (promoter expresses at 0.16× of wild-type <i>TEF</i>)	This study
pSH47	pRS416 expressing <i>cre</i> from <i>GALI</i> promoter	(Gueldener et al., 2002)
pPL5071	pRS416 expressing <i>cre</i> from <i>TEF1</i> * promoter	This study
pPL5608	pRS414 expressing <i>cre</i> from <i>TEF1</i> * promoter	This study
pPL5606	pRS41- <i>ADE2</i> expressing <i>cre</i> from <i>TEF1</i> * promoter	This study
pPL5628	pRS41- <i>MET15</i> expressing <i>cre</i> from <i>TEF1</i> * promoter	This study
pUG27	pRS400 series backbone containing <i>loxP</i> flanked <i>Sp^ψhis5⁺</i> gene driven from <i>Ag[§]TEF1</i> promoter	(Gueldener et al., 2002)
pUG72	pRS400 series backbone containing <i>loxP</i> flanked <i>URA3</i> gene driven from <i>Ag[§]TEF1</i> promoter	(Gueldener et al., 2002)
pPL5617	pRS400 series backbone containing <i>loxP</i> flanked <i>pac</i> gene driven from <i>Ag[§]TEF1</i> promoter	This study
pPL5618	pRS400 series backbone containing <i>loxP</i> flanked <i>DHFR</i> * gene driven from <i>Ag[§]TEF1</i> promoter	This study

Sp^ψ = *Schizosaccharomyces pombe*Ag[§] = *Ashbya gossypii*

Table 3
Plasmid request information

Plasmid	Short Description	GenBank Accession #	Addgene Plasmid #
pPL5617	<i>loxP-puro^r-loxP</i> PCR template	KP159511	60928
pPL5618	<i>loxP-puro^r-loxP</i> PCR template	KP159512	60929
pPL5071	<i>URA3</i> marked <i>TEF1[*]-cre</i>	KP159513	60930
pPL5608	<i>TRP1</i> marked <i>TEF1[*]-cre</i>	KP159514	60931
pPL5606	<i>ADE2</i> marked <i>TEF1[*]-cre</i>	KP159515	60932
pPL5628	<i>MET15</i> marked <i>TEF1[*]-cre</i>	KP159516	60933