Integrative modules for efficient genome engineering in yeast

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ABSTRACT  We present a set of vectors containing integrative modules for efficient genome integration into the commonly used selection marker loci of the yeast Saccharomyces cerevisiae. A fragment for genome integration is generated via PCR with a unique set of short primers and integrated into HIS3, URA3, ADE2, and TRP1 loci. The desired level of expression can be achieved by using constitutive (TEF1p, GPD1p), inducible (CUP1p, GAL1/10p), and daughter-specific (DSE4p) promoters available in the modules. The reduced size of the integrative module compared to conventional integrative plasmids allows efficient integration of multiple fragments. We demonstrate the efficiency of this tool by simultaneously tagging markers of the nucleus, vacuole, actin, and peroxisomes with genomically integrated fluorophores. Improved integration of our new pDK plasmid series allows stable introduction of several genes and can be used for multi-color imaging. New bidirectional promoters (TEF1p-GPD1p, TEF1p-CUP1p, and TEF1p-DSE4p) allow tractable metabolic engineering.

INTRODUCTION
Saccharomyces cerevisiae is an indispensable tool for high throughput studies of biological processes. The ever-increasing diversity of tools for yeast genome manipulation allows systematic examination of biological pathways in a highly physiological, cost-effective, and tractable manner [1-7]. However, attempts to introduce multiple genes into chromosomal loci often encounter complications due to decreased efficiency and cloning limitations, such as overlaps in restriction site usage amongst multiple inserts [7, 8]. In order to overcome the difficulties of multiple gene integrations we sought to expand the variety of yeast integration cassettes with a specially designed pDK vector set.

Several methods enable rapid gene introduction. The most common ones are ectopic plasmid expression and chromosomal integration. Ectopic expression via multicopy or centromeric plasmids is often faster and easier than integration, but poses problems due to highly inhomogeneous expression [9, 10]. Hence, multi-color imaging and metabolic engineering in yeast often require stable integration of genes. Genome integration via homologous recombination has advantages over ectopic expression: e. g. stable strains, controlled copy number, and uniform expression [9].

Several integration strategies are commonly employed. (1) Yeast integrative plasmids are introduced into the genome in linearized form [1]. The linearized fragment contains the desired insert and selection marker, but also substantial superfluous genetic material including the bacterial selection marker and replication origins [1]. These extraneous sequences may reduce integration efficiency [8]. Additionally, the customized insert must not contain the restriction site used for plasmid linearization, adding further limitations. (2) Another frequently used strategy is PCR amplification using extended primers with homology to chromosomal loci rather than selective markers does not pose a particular advantage since the selective marker locus still has to be present in the integrated fragment. (3) Recent advances in CRISPR/Cas9 genome engineering allow highly efficient introduction of multiple fragments with short homology regions and often without the need for a selective marker, however the strain must...
We also include 4 bidirectional promoters (Figure 1A). Constructed novel bidirectional promoter sets to maximize available markers we also carry Cas9 and gRNA expressing cassettes [4, 11, 12]. (4) I-SceI-assisted integration has a significantly increased efficiency, but this approach also requires the additional expression of the meganuclease I-SceI locus [13]. (5) Plasmids carrying integrative cassettes excised by restriction with extended homology regions flanking the insert significantly improve integration efficiency [6, 9, 14]. We were inspired by this last strategy and decided to create a set of yeasts integration cassettes that contain extended homology regions corresponding to a common selectable marker locus, which eliminates the need to have a marker locus inside the cassette. An advantage of not having a selectable marker locus inside the cassette reduces its size and potentially increases integration efficiency [8]. To reduce multiple integration time and to maximize available markers we constructed novel bidirectional promoter sets (Figure 1A).

The pDK series includes 24 plasmids which carry an integrative module for 4 common genetic markers (HIS3, URA3, ADE2, and TRP1). Constitutive (TEF1p, GPD1p), daughter specific (DSE4p) and inducible (CUP1p and bidirectional GAL1/10p) promoters flank multiple cloning sites. We also include 4 bidirectional promoters (GAL1p-GAL10p, TEF1p-GPD1p, TEF1p-CUP1p, and TEF1p-DSE4p) which allow multiple integrations (Figure 1, Table 1). PCR is carried out with short primers specific to a selectable marker in order to integrate the module (see supplemental Table 1). The pDK set can be used for multi-color imaging, metabolic engineering, or any set of experiments that require stable integration, and stability of the insert. 24 integrative modules of pDK series were transformed into the W303 strain. pDK integrative modules have comparable integration efficiencies (Supplemental Figure 1A). Although double integration is possible we recommend sequential integration and using bidirectional promoters to expedite the work flow. We also compared the integration efficiency to other integration strategies: (1) pRS series – conventional linearized integrative plasmids [1], (2) EasyClone strategy – extended homology regions, excisable marker and restriction based integration [6], and (3) extended primers - 45bp homology region. In general, extended homology region based strategies provide higher efficiency of integration. pDK series integration is more effective than pRS series, and is comparable with EasyClone efficiency, which also has a reduced insert size but relies on restriction based integration.

Integrative modules allow stable and efficient integration of multiple inserts
pDK vectors were examined for integration efficiency, correct integration, and stability of the insert. 24 integrative modules of pDK series were transformed into the W303 strain. pDK integrative modules have comparable integration efficiencies (Supplemental Figure 1A). Although double integration is possible we recommend sequential integration and using bidirectional promoters to expedite the work flow. We also compared the integration efficiency to other integration strategies: (1) pRS series – conventional linearized integrative plasmids [1], (2) EasyClone strategy – extended homology regions, excisable marker and restriction based integration [6], and (3) extended primers - 45bp homology region. In general, extended homology region based strategies provide higher efficiency of integration. pDK series integration is more effective than pRS series, and is comparable with EasyClone efficiency, which also has a reduced insert size but relies on restriction based integration.

To evaluate integration stability, a GFP reporter was cloned into the pDK series. The stability of the integration was tested by avoiding selection for more than 80 generations and then counting fluorescent cells (Supplemental Figure 1B). Modules are integrated with 95 - 100% accuracy into the marker region as verified by PCR (Supplemental

### RESULTS AND DISCUSSION

#### Vector overview
We designed 24 pDK plasmids for stable expression of multiple genes in the yeast *S. cerevisiae* (Figure 1, Table 1). Vectors can be used for stable integration into 4 common *S. cerevisiae* selective marker loci HIS3, URA3, ADE2, and TRP1. Plasmid variations include 6 promoters (Figure 1C): Constitutive TEF1p, inducible CUP1p, and inducible bidirectional GAL1/10p in opposing orientation, constitutive bidirectional TEF1-GPD1p, constitutive-inducible bidirectional TEF1-CUP1p, constitutive-daughter-specific bidirectional TEF1-DSE4p (bidirectional promoter sets have two multiple cloning sites (MCS) in opposing orientation). The markers are split into two parts (see plasmid construction for details) and are flanking the region containing a promoter, MCS, and a terminator (Figure 1A). The fragment is amplified with primers specific to the marker (Supplemental Table 1). The orientation of the split markers allows integration of amplified region in a traditional homologous recombination driven way, resulting in doubling of the marker region (Figure 1B).

#### Table 1. pDK plasmid series with integrative modules.

| Module | HIS3 | URA3 | TRP1 | ADE2 |
|--------|------|------|------|------|
| TEF1p-MCS-TEF1t | pDK-HT | pDK-UT | pDK-TT | pDK-AT |
| CUP1p-MCS-TEF1t | pDK-HC | pDK-UC | pDK-TC | pDK-AC |
| GAL1p-MCS(1)-CYC1t GAL10p-MCS(2)-ADHt | pDK-HGG | pDK-UG | pDK-TG | pDK-AG |
| TEF1p-MCS(1)-CYC1t GPD1p-MCS(2)-ADHt | pDK-HTG | pDK-UTG | pDK-TTG | pDK-ATG |
| TEF1p-MCS(1)-CYC1t CUP1p-MCS(2)-ADHt | pDK-HTC | pDK-UTC | pDK-TTC | pDK-ATC |
| TEF1p-MCS(1)-CYC1t DSE4p-MCS(2)-ADHt | pDK-HTD | pDK-UTD | pDK-TTD | pDK-ATD |
Figure 1B). Marker based integration is advantageous because it provides accurate targeting compared to the CRISPR/Cas9 strategy [15]. We also tested the stability of multiple integrations, since many of them carry same promoters and/or terminators, which can potentially lead to a loop-out of the fragments. Single and multiple integrations provide reliable homogenous expression levels under non-selective conditions (Supplemental Figure 1B, C).

Application of pDK plasmids to multi-color imaging reveals order of organelle inheritance

As proof of concept for multi-color imaging we constructed peroxisomal, nuclear, and actin [16] markers using the pDK vector series and used the strain for 3D time-lapse microscopy (4D imaging) (Figure 2) [17]. Nuclear localization signal fused to a far-red fluorophore was used to visualize the nucleus. Peroxisome localization signal - tripeptide SKL was fused to the mCherry C-terminus to visualize peroxisomes. We also used LifeAct fused to GFP to visualize actin in living yeast [16]. The vacuolar marker VPH1 was endogenously tagged with mBFP or GFP. Peroxisomes are known to use actin cables for inheritance [18]. Inp2, an integral peroxisomal membrane protein, binds the Myo2 motor ensuring localization to the bud [19, 20]. Most of the organelles in yeast use actin cables for bud trafficking during division [21]. To determine whether organelle inheritance proceeds in a parallel or a serial way we examined the inheritance of the vacuole, peroxisomes, and the nucleus (Figure 2, Supplemental movie “wtdivision”). The nucleus itself is inherited in a microtubule dependent manner, however the astral microtubules are delivered on actin to the bud [22]. Multi-color imaging revealed that the first instance of peroxisome inheritance happens in parallel with the vacuole in the first 15 min of cell division (Figure 2B). Deletion of INP2 abolishes peroxisome inheritance, which has no effect on the time of vacuole inheritance [19] (Figure 2C, Supplemental movie “inpdivision”). Interestingly, the nucleus is inherited prior to the end of cell division. The integrative vector series that we have developed allows for efficient integration of up to 8 markers, enabling us to image cellular compartments simultaneously.
FIGURE 2: Proof of concept - Organelle inheritance order during division. (A) In vivo 4-color imaging of peroxisome (SKL signal C-terminally fused to 4 mCherry (mCH) on pDK-UT), actin (LifeAct fused to GFP on pDK-AT), nucleus (SV40 nls signal fused to 3 far-red fluorophores on pDK-HT), and vacuole (VPH1 endogenously tagged with mBFP), the scale bar is 1 µm. The graph represents proximity of peroxisome to actin cable during division, Fluorescence Intensity (FI) was calculated along the x axes (arrow on the merge image) for red (peroxisome) and green (actin) channels. (B) The timeline of organelle inheritance in the wild type (wt) strain, scale bar is 1 µm, time points represent the average (n = 30) time of organelle inheritance from the start of division, strain carries SKL signal C-terminally fused to 4 mCherry (mCH) on pDK-UT module, SV40 nls signal fused to 3 far-red fluorophores on pDK-HT module, and VPH1 endogenously tagged with GFP. (C) The timeline of the organelle inheritance in the Δinp2 strain, scale bar is 1 µm.
Application of pDK plasmids to multi-copy integration

As proof of concept of exquisitely controlled inducible expression of a gene using multiple integrations we inserted GFP tagged VHL under inducible CUP1 promoter in 4 marker loci. The von Hippel Landau tumor suppressor (VHL) has often been used as a model substrate to study protein aggregation in yeast [23]. Carefully controlling expression levels of integrated proteins is essential to many studies. In the case of VHL and similar proteins, increased protein concentration results in gradual protein self-association with subsequent decrease in the monomer fraction in vitro. This leads to the formation of protein inclusions or aggregates. To test if there is a correlation of misfolded protein concentration and inclusion formation in vivo we introduced 1-4 copies of GFP-VHL under CUP1p (Figure 3A, B) in yeast. We scored inclusion formation as a function of concentration and temperature. Aggregation strongly correlates with an increase in temperature, \( r(\text{temperature}) = 0.76 \) (\( p < 0.05 \)), but has no significant correlation with VHL concentration, \( r(\text{concentration}) = 0.005 \) (\( p = 0.98 \)). This could indicate a number of things. One interpretation of these data is that, regardless of the concentration, GFP-VHL forms a small percentage of overall protein content in inclusions (because of the abundance of misfolded and unstructured proteins in yeast). Another possibility is that without significant heat shock the quality control system is able to degrade a spectrum of misfolded VHL concentrations, but at higher temperatures it becomes uniformly inhibited. Additional experiments could make similar titrations of proteasome and chaperone activity. Together, these data are compelling as proof-of-concept for multi-gene integrations.

Bidirectional promoter sets

To improve the time of strain construction, we built versatile bidirectional promoter sets, allowing integration of two inserts under different promoters at the same time. The promoters are positioned in the opposite orientation (Figure 1A). Previously described GAL1p/GAL10p and 3 novel promoters: TEF1p-GPD1p, TEF1p-CUP1p, and TEF1p-DSE4p

![Figure 3](image-url)

**FIGURE 3: Proof of concept - Concentration does not correlate with inclusion formation.** (A) Gradual increase in the amount of GFP-VHL according to integrated copies, Fluorescence Intensity was quantified in 30 single cells in the population, the average and standard errors are represented on the graph. (B) Confocal images of cells carrying 4 copies of GFP-VHL on pDK-AC, TC, UC, HC modules subjected to the range of temperatures for 1h, scale bar 1 µm. (C) Quantification of inclusion forming cells in the population according to the change in the temperature and the concentration, the numbers represent % of the cells with inclusions (n = 300), correlation coefficient of aggregation and temperature \( r(\text{temperature}) \) and aggregation and concentration \( r(\text{concentration}) \) is provided under the diagram.
were introduced into pDK series. To validate promoter sets we constructed GFP/mCherry reporters and visualized the cells during the log phase (Figure 4). Bidirectional promoters allow controlled inducible, semi-inducible, constitutive and daughter-specific expression (Figure 4A-D). Not only do they facilitate strain construction, bidirectional sets can also be used for cell sorting (daughter/mother cells), studying aging, or screening yeast genome collections [24, 25].

In summary, the pDK vector series allows for efficient multiple integrations and thus is a useful tool for multicolor imaging, metabolic engineering, controlled expression of genes of interest, and stable yeast strain production. We therefore hope that pDK vectors will be a useful tool for the yeast community.

**FIGURE 4: Bidirectional promoter reporters.** (A) Galactose inducible bidirectional promoter, cells carrying pDK-HGG-GFP-mCherry were induced with galactose or grown on glucose for 6 hours, scale bar 1 µm. (B) Constitutive-inducible promoter, cells carrying pDK-HTC-GFP-mCherry were grown with and without (control) copper2+ for 4 hours, scale bar 1 µm. (C) Constitutive bidirectional promoter, cells carrying pDK-HTG-GFP-mCherry were grown on glucose containing medium to middle log phase, scale bar 1 µm. (D) Daughter-specific-constitutive bidirectional promoter, cells carrying pDK-HTD-GFP-mCherry module were grown on glucose containing medium on the microscope, frames are shown (0 min, 60 min, 100 min). (E) Comparison of fluorescence intensity of bidirectional reporters, the graph shows the average of fluorescence intensity of 20 cells and standard error.
MATERIALS AND METHODS

Strains and media

We used standard conditions for cultivating yeast and bacterial cells [26]. Yeast were grown in the selective medium (1.7 g/l yeast nitrogen base without amino acids and ammonium sulfate (Difco Laboratories), 5 g/l ammonium sulphate, 0.77 g/l complete, 2 g/l amino acids supplement powder mix [27], 20 g/l glucose, and 20 g/l agar for the solid medium) or rich medium (20 g/l Peptone, 10 g/l yeast extract, 20 g/l glucose, and 20 g/l agar for the solid medium). Galactose induction was performed on selective medium supplemented with 20 g/l galactose instead of glucose for 6 hours. CUP1p induction was performed by addition of 50 µM Cupric Sulphate to the selective medium and growing cells for 4 hours. Plasmids were constructed using Escherichia coli strain DH5α. Yeast strain W303-1B (MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15) was used for the experiments.

Plasmid construction

pUC19 plasmid [28] was used as a backbone for pDK vectors. Primers used in this study are summarized in supplemental tables: 1 - primers used for yeast integration, 2 - primers used for construction of plasmids. Markers loci (including promoters and terminators) were split in the following parts: HIS3 (after 286bp of the ORF), TRP1 (after 224 bp of the ORF), ADE2 (after 462 bp of the ORF), and URA3 (after 420 bp of the ORF). Split fragments were amplified with: HIS3F1/R1, TRP1F1/R1 and TRP1F2/R2, ADE2F1/R1 and ADE2F2/R2, and cloned into pUC19’s TEF1p/ HindIII and PfoI/EcoRI, EcoRI/PfoI and PciI/HindIII, HindIII/PciI and PfoI/EcoRI, HindIII/PciI and PfoI/EcoRI respectively, resulting in the plasmids pDK-HH, pDK-UT, and pDK-TT, and pDK- AA. Promoters and terminators (TEF1p, CUP1p, TRP1p) were amplified with: TEF1PF/R, CUP1PF/R, TEF1TF/R primers and cloned into pUC19’s TEF1p/ HindIII and PfoI/EcoRI, EcoRI/PfoI and PciI/HindIII, HindIII/PciI and PfoI/EcoRI, HindIII/PciI and PfoI/EcoRI respectively, resulting in the plasmids pDK-HH, pDK-UT, and pDK-TT, and pDK- AA. Promoters and terminators (TEF1p, CUP1p, TRP1p) were amplified with: TEF1PF/R, CUP1PF/R, TEF1TF/R primers and cloned into pUC19’s NdeI/EcoRI, NdeI/EcoRI, and SalI/HindIII sites, respectively, resulting in pUC19-TEF1p-TEF1t and pUC19-CUP1p-CUP1t plasmids. TEF1p-TEF1t and GAL1/10p-ADH1/CYC1t modules were amplified from previously constructed pUC19-TEF1p-TEF1t and pUC19- CUP1p-CUP1t plasmids, and pESC-URA (Agilent) vectors using the primers: CUPTEFHHF, CUPTEUFUUF, CUPTETTTTF, CUPTETFRAF and CUPTETFR for TEF1p-TEF1t and, CUP1p-CUP1t modules, and GALHHF, GALUHF, GALTTF, GALAFAF and GALR for GAL1/10p-ADH1/CYC1t module, and cloned using Gibson Assembly mix (NEB) into pDK-HH, pDK-UU, pDK-TT, and pDK-AA vectors linearized with EcoRI/SalI restriction enzymes resulting in 12 pDK plasmids. Plasmid names contain the first letter of the marker followed by the first letter/s of the promoter/s (Table 1). TEF1 promoter was amplified with TEFbF/R primers and cloned into pESC-URA Agel/BamHI sites resulting in the pESC-TEFp plasmid. CUP1, DSE4, and GPD1 promoters were amplified with CUPbF/R, DSEbF/R, and GPDbF/R primers and cloned into the pESC-TEFp vector into Agel/EcoRI sites resulting in pESC-TG, TC and TD plasmids carrying bidirectional promoters. Bidirectional promoters were subcloned using BamHI/EcoRI into pDK-HG/UG/TG/AG, resulting in 12 bidirectional promoter sets (Table 1).

The nuclear cellular marker plasmid was constructed by cloning the SV40 nuclear localization signal fused to (mCherry)4 [29] into pDK-HT vector EcoRI/SmoI sites. The peroxisome marker was constructed by cloning the (mCherry)A-SKL sequence into pDK-UT plasmid. Actin was visualized via the LifeAct fragment fused to GFP [16], LifeAct-GFP was subcloned between BamHI/EcoRI sites into pESC-TG, TC, and TD plasmids carrying bidirectional promoters. Bidirectional promoters were subcloned using BamHI/EcoRI into pDK-HG/UG/TG/AG, resulting in 12 bidirectional promoter sets (Table 1).

For endogenous tagging we modified the pKT127 [30]...

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**TABLE 2.** Strains used in this study.

| Strain     | Genotype                          |
|------------|-----------------------------------|
| BY4741     | MATa his3Δ0 leu2Δ0 met15Δ0 ura3Δ0 |
| W303α      | MATα leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15 |
| yDK- inp2, nuc, pex, vac | W303α inp2Δ::kanMX his3-11,15:::ns-::(tagRFP657):::HIS3 ura3-1::(mCherry)3-SKL:::URA3 VPH1-GFP-HPH |
| yDK- nuc, pex, vac | W303α his3-11,15::::ns-::(tagRFP657):::HIS3 ura3-1::(mCherry)3-SKL:::URA3 VPH1-GFP-HPH |
| yDK- nuc, pex, la, vac | W303α his3-11,15::::ns-::(tagRFP657):::HIS3 ura3-1::(mCherry)3-SKL:::URA3 ade2-1::::LifeAct-GFP:::ADE2 VPH1-mBFP-HPH |
| yDK - HHCTGV | W303α his3-11,15::::CUP1p-GFP-VHL-TEF1t:::HIS3 |
| yDK – HHCTGV, UUCTGV | W303α his3-11,15::::CUP1p-GFP-VHL-TEF1t:::HIS3 ura3-1::::CUP1p-GFP-VHL-TEF1t:::URA3 |
| yDK – HHCTGV, UUCTGV, TTCTGV | W303α his3-11,15::::CUP1p-GFP-VHL-TEF1t:::HIS3 ura3-1::::CUP1p-GFP-VHL-TEF1t:::URA3 trp1-1::::CUP1p-GFP-VHL-TEF1t:::TRP1 |
| yDK – HHCTGV, UUCTGV, TTCTGV, AACTGV | W303α his3-11,15::::CUP1p-GFP-VHL-TEF1t:::HIS3 ura3-1::::CUP1p-GFP-VHL-TEF1t:::URA3 trp1-1::::CUP1p-GFP-VHL-TEF1t:::ADE2 |
plasmid by inserting the mBFP sequence or GFP sequence into Pocl/Acs1 sites and HPH marker sequence into BglII/Plm1 sites. GFP reporters were constructing by cloning GFP into SacI/Xmal sites of pDK-HT/HC, and BamHI/Xmal sites of pDK-HGG with primers GFPxR, GFPbF, GFPsF and subcloning the fragment containing the marker across different marker plasmids and bidirectional promoter ones. mCherry was amplified from a corresponding plasmid with a set of primers listed in Table 2 using the following parameters (95°C- 5', [95°C- 30'', 62°C- 30'' (increment 0.8°C per cycle), 72°C- X min (X = length of the fragment in kb)]25 cycles, 72°C- X min (X = length of the fragment in kb))25 cycles, and high fidelity polymerase generating blunt-end products, e.g. KAPA HiFi DNA Polymerase (KAPA Biosystems). A PCR protocol with fixed primer binding time can also be used. Up to 2 µg of PCR product is transformed using LiAc/PEG transformation [32] with modifications – 50 µl of DMSO is added prior to heat shock, heat shock time is reduced to 15 min at 42°C. 20 random clones carrying integrative modules were verified by PCR.

Stability of integration
Yeast strains were grown in rich medium in three replicates. The culture was diluted 1/100 every 24 hours for 10 days. The culture was analyzed by reporter fluorescence on the first and tenth day.

Confocal Microscopy
For imaging yeast cells were grown to mid-log phase and seeded on concanavalin A (Sigma) coated 4-well microscope plates (IBIDI). For induction galactose rich media (division experiments, Figure 2) or minimal media with 50 µm Cu added prior to heat shock, heat shock time is reduced to 15 min at 42°C. 20 random clones carrying integrative modules were verified by PCR.

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