Development of an Assembly and Delivery Platform for DNA Circuit Construction in *Saccharomyces cerevisiae*

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Background and summary

Complex behavior within eukaryotic cells manifest from layered regulatory networks changing the transcription of many genes. To systematically study these pathways by modulating individual components—or in the case of synthetic biology, building new network architectures by creating DNA circuit—it is critical to control multiple genes simultaneously under tightly regulated or inducible expression. In the case of network construction in *Saccharomyces cerevisiae*, there has been a lack of both suitable, well-characterized parts (promoters and regulators) as well as a standardized platform for DNA assembly and delivery of gene circuits. Here, we present a framework for building gene circuits as well as a set of fully characterized DNA parts for use in *Saccharomyces cerevisiae*. The entire procedure of building a gene circuit from more than 10 basic parts took less than 5 days with only a workload of 1-3 hours per day. A diverse promoter collection comprising five different types was generated: constitutive, yeast native inducible, synthetic inducible, synthetic promoters regulated by activators, and synthetic promoters regulated by repressors. Altogether, the range of promoters span 2-fold to 105-fold expression above the background, the new inducible systems allow 11-fold change in expression, and the activators/repressors show a maximum 35-fold and 45-fold change of expression. This study demonstrates the feasibility for the quick and easy construction of gene circuits for delivery into *S. cerevisiae* and the utility of a fully characterized set of diverse promoters, activators, and repressors. This assembly system combined with DNA parts will be useful for constructing large-scale gene circuit libraries with reliable gene expression and for designing logic operations for a complex network in *S. cerevisiae.* Moreover, we anticipate that our system will allow for the controlled study of multi-step pathways by enabling manipulation of single protein expression.

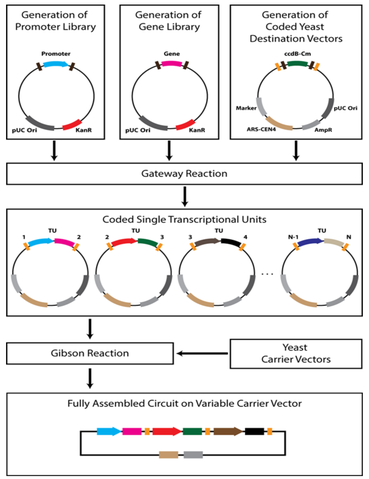

Figure 1. Two-stage assembly system for eukaryotic circuit construction and delivery

Toolkit development

To ease construction of genetic networks consisting of multiple transcriptional units (TUs) and to allow flexible DNA delivery (including low-copy & high-copy plasmids or site-specific integration), we created a two-step platform utilizing Gateway® (Thermo Fisher Scientific, Inc.) recombination-based cloning and Gibson Assembly® (Synthetic Genomics, Inc.) to permit circuit construction. To build any network, the following steps are undertaken:

1. Researcher designs the network (*i.e.* what genes, expression levels, regulation needed).
2. Appropriate promoters/genes are obtained from the ATCC Yeast Toolkit, the NIH plasmid repositories, or are cloned.
3. Single TUs are assembled via LR recombination with user-directed 'position' vector to form TU1, TU2, TU3, *etc*. (that can be used in yeast for component testing).
4. Individual TUs are linearized with I-SceI, combined with a carrier vector (high-copy, low-copy, or site-specific integration) and adapter fragment via Gibson Assembly® to form complete multi-TU networks that are ready for yeast transformation.

Components of ATCC yeast synthetic biology toolkit

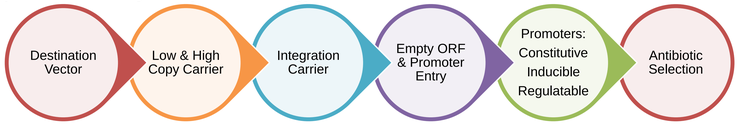

Table 1: Comparison to other DNA assembly platforms

	Gateway	Gibson	Golden-Gate	Synthesis	ATCC Toolkit
Efficiency of *in vitro* steps	Yes	Maybe	Size limits	Maybe	Yes
No PCR Mutation Risk	Yes	Maybe	Maybe	Yes	Yes
Network Variants	N/A (1-T.U.)	Yes	Yes	$ Limited	At 'Parts' Level
Existing Parts Repositories	Yes	No	Limited	N/A	Yes
Flexible Delivery	N/A (1-T.U.)	Yes	Yes	Yes	Yes
Large (>6 T.U.s)	No	Yes	Hierarchical	Yes	Yes
Scarless	No	Yes	Yes	Yes	No
Single T.U. Testing	Yes	Maybe	Yes	Maybe	Yes

Initial validation of construction and assembly stages

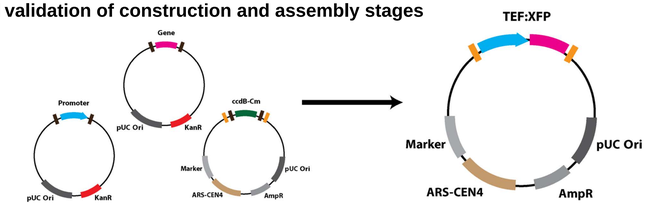

Figure 2. Generation of a single TU comprising four different fluorescent protein genes via Gateway® reaction. A common URA selection destination vector was combined with pTEF to form 4 unique expression vectors with constitutive eCFP, eGFP, eYFP, or mKate expression. Individual TUs were assembled from library stocks to verified yeast colonies in 96 hours.

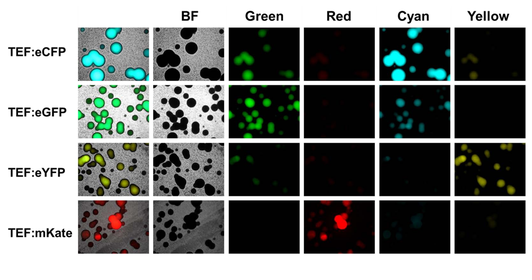

Figure 3. Single-stage LR product verification. Four plasmids were simultaneously constructed on a low-copy plasmid with uracil auxotrophic selection differing only in the fluorescent protein chosen. Plasmids were transformed into yeast with W303-A background prior to imaging using a Zeiss Axiovert 200m.

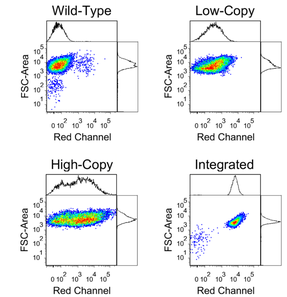

Figure 4. Two-stage assembly LR and Gibson product characterization. mKate and kanMX transcriptional units were formed into 3 markers differing in backbone: a low-copy, high-copy, or HO-locus integrating plasmid. The assays were performed after 16 hours under G-418 selection to ensure the measurement is at the steady-state.

Context-dependent characterization of constitutive promoters

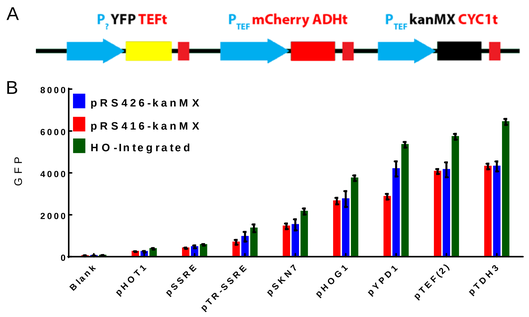

Figure 5. Constitutive promoter characterization. A) Schematic outline of three TUs that were assembled with 8 different constitutive promoters (P_2) that were used to drive the expression of YFP prior to assembly. B) All promoters are normalized for expression using mCherry and calibrated beads under G418 selection in W303-A background. Geometric means are reported and error bars correspond to n=3 biological replicates.

Characterization of DOX-inducible promoter systems

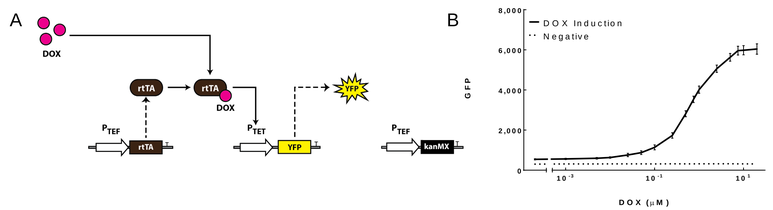

Figure 6. DOX inducible circuit and characterization. A) Schematic diagram of DOX-inducible system. Three TUs were assembled with constitutive expression of rtTA, inducible expression of YFP, and KanMX selection. B) Dose response of DOX on the rtTA-dependent YFP expression. All promoters are normalized using calibrated beads and geometric means. Error bars correspond to n=3 biological replicates.

Generation and characterization of two novel small molecule induction systems

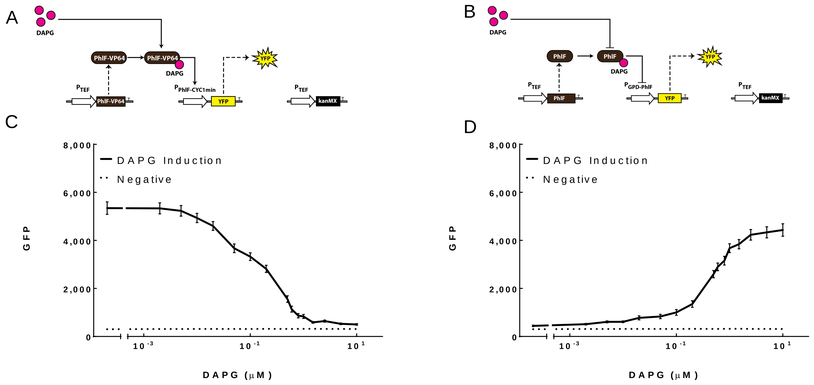

Figure 7. DAPG inducible circuit and characterization. A-B) We created two additional small-molecule inducible systems for use in yeast. We relied on the PhlF repressor, which is a TetR homlog that binds the small molecule 2,4-diacetylphloroglucinol (DAPG). By fusing PhlF operator sites downstream of the constitutively active GPD promoter in tandem with PhlF expression, or fusing PhlF operator sites upstream of the CYC1min promoter in tandem with a PhlF-VP64 fusion protein, we were able to make two inducible systems. The TUs were assembled with constitutive expression of rtTA, inducible expression of YFP, and kanMX selection. C-D) The dose response of DAPG on YFP expression under regulation of $P_{PhlF\text{-}CYC1min}$/PhlF-VP64 and $P_{GPD\text{-}PhlF}$/PhlF, respectively. All promoters are normalized using calibrated beads and geometric means. Error bars correspond to n=3 biological replicates.

Characterization of transcriptional regulators

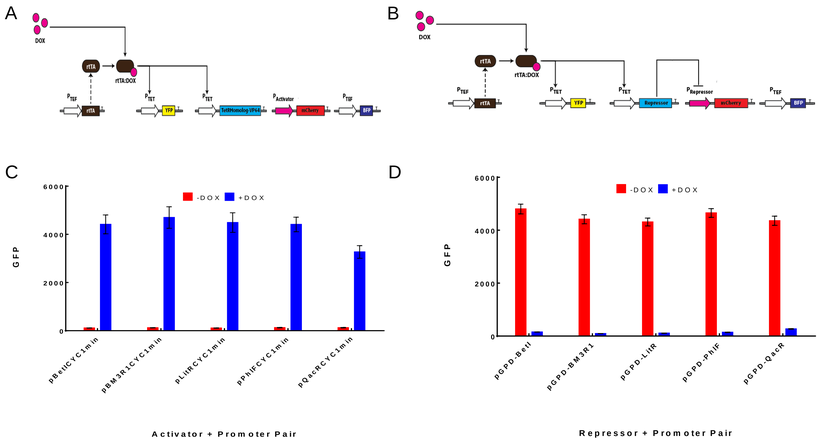

Figure 8. Transcriptional regulator characterization. A-B) Similar to construction of the PhlF inducible systems, we created a set of promoters with TetR homolog binding sites in conjunction with expression of their TetR or TetR-VP64 homologs. To characterize them, in the presence of DOX, rtTA binds the pTET promoter creating expression of both YFP and the regulator of interest. C-D) The regulator can then bind it's cognate promoter, modulating mCherry expression that is measured and normalized to MEFLs.

Conclusions

- We created a toolkit that encompasses 47 plasmids and is readily expandable to be used in creating genetic networks with up to 8 individual transcriptional units in less than 5 days with only a workload of 1-3 hours per day.
- The toolkit includes a promoter collection that spans 2-log decades of constitutive protein expression and novel inducible systems that span 11-fold expression change.
- The toolkit includes set of transcriptional regulators capable of ~40-fold change differences in regulation.