Supporting Information for

Automated continuous evolution of proteins *in vivo*

Ziwei Zhong[1,*], Brandon G. Wong[2,3,*], Arjun Ravikumar[1,2,3], Garri A. Arzumanyan[1], Ahmad S. Khalil[2,3,4,#], and Chang C. Liu[1,5,6,#]

[1]Department of Biomedical Engineering, University of California, Irvine, California, USA.

[2]Biological Design Center, Boston University, Boston, Massachusetts, USA.

[3]Department of Biomedical Engineering, Boston University, Boston, Massachusetts, USA.

[4]Wyss Institute for Biologically Inspired Engineering, Harvard University, Boston, Massachusetts, USA.

[5]Department of Chemistry, University of California, Irvine, California, USA.

[6]Department of Molecular Biology & Biochemistry, University of California, Irvine, California, USA.

*These authors contributed equally to this work.

[#]Correspondence to: Chang C. Liu (ccl@uci.edu) and Ahmad S. Khalil (khalil@bu.edu)

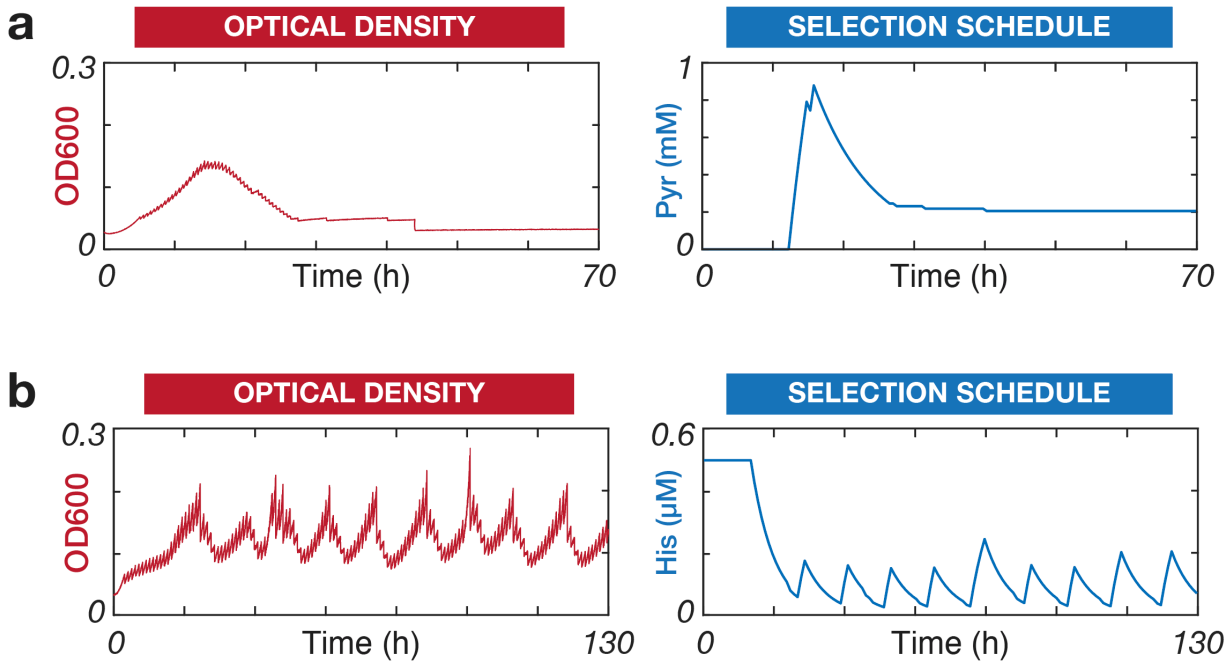

Figure S1. Sample evolution experiments in eVOLVER using the control algorithm derived from Toprak et al., 2013.[19] Briefly, this algorithm sampled OD at fixed intervals and if (1) the current OD is greater than a threshold and (2) if the current OD is greater than the previous OD, the growing cultures were diluted with the selection media. Otherwise, the culture would be diluted with the base media **(a)** An example of *Pf*DHFR evolution using the Toprak *et al.* algorithm. ZZ-Y435 was inoculated into eVOLVER and grown as described in **Methods** except the control algorithm was as described in Toprak *et al.*[19] The OD of the adapting culture (red) and the calculated concentration of pyrimethamine (blue) are shown. After the initial increase and subsequent decrease of pyrimethamine, no further growth is seen in 40 hours. **(b)** An example of *Tm*HisA evolution using the Toprak *et al.*[19] algorithm. ZZ-Y323 was inoculated into eVOLVER and grown as described in **Methods** except for the control algorithm. The OD of the adapting culture (red) and the calculated concentration of histidine in the media (blue) are shown. The concentration of histidine is observed to oscillate during selection and is unable to successfully adapt.

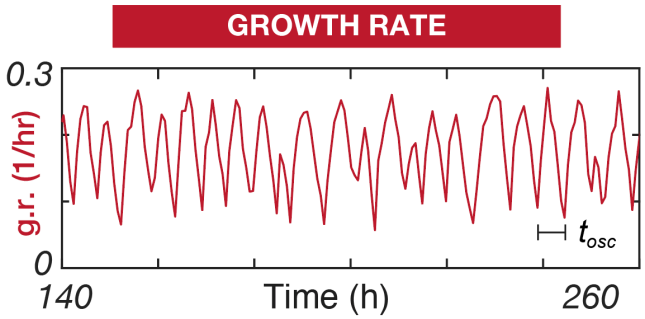

Figure S2. Oscillation of growth rate of ZZ-Y323 during the empirical determination of PID settings. ZZ-Y323 was inoculated into eVOLVER and SC-UL was used as the base media and SC-ULH was used as the full adaptation media. K_P was iterated from 0 to 4 over 120 hours while K_I and K_D were set to zero. Oscillations were observed when $K_P = 4$, and the period and amplitude of the oscillation were used to estimate the parameters for the PID control algorithm.

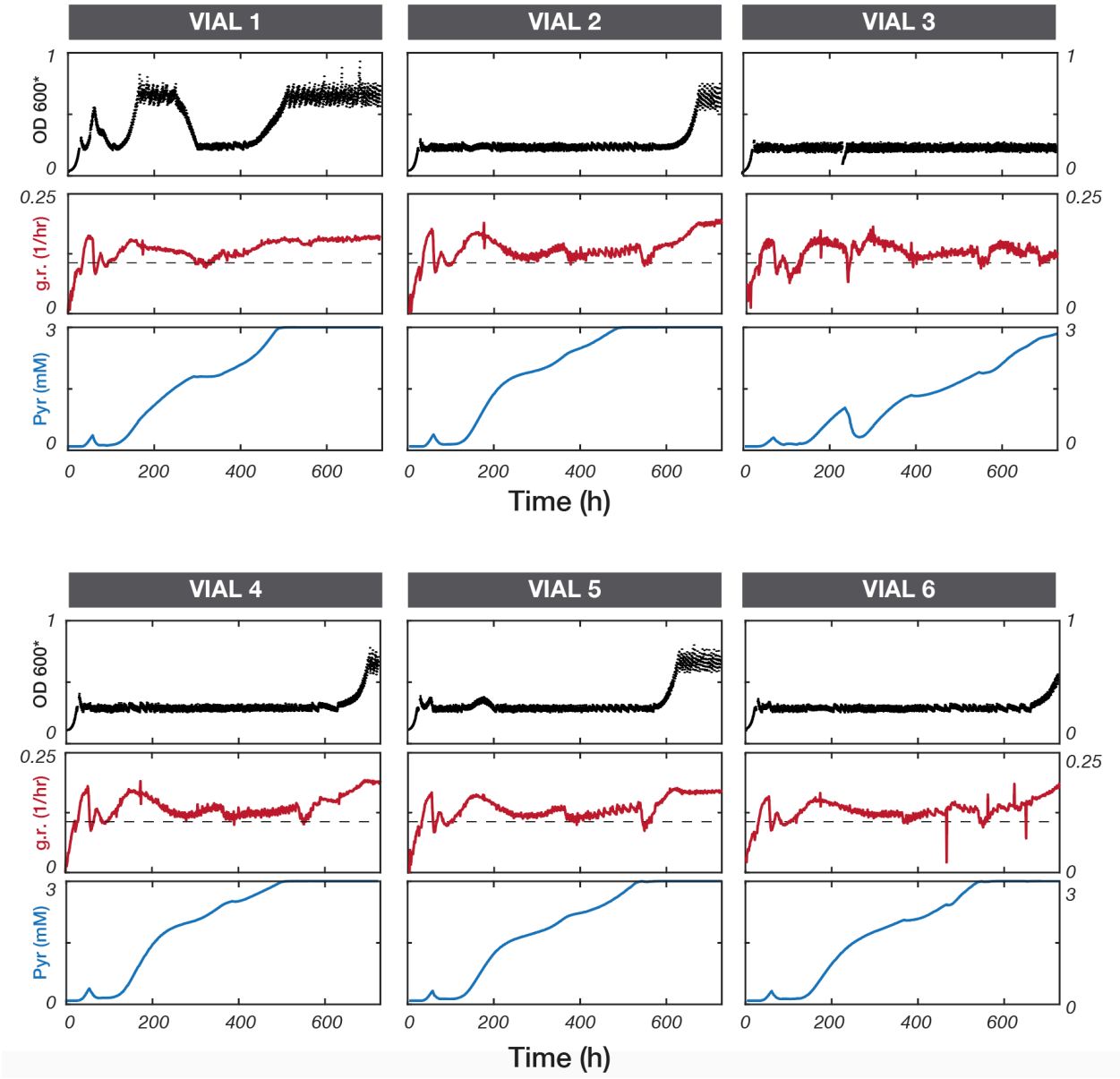

Figure S3. Adaptation history for all six replicates during *Pf*DHFR evolution. OD (black dot), growth rate (red line), target growth rate (black dash), and pyrimethamine concentration (blue line) are plotted for all six independent replicates. All six independent cultures were able to adapt to 3 mM pyrimethamine without the need for pre-programmed selection schedules or user intervention except to replenish media stocks.

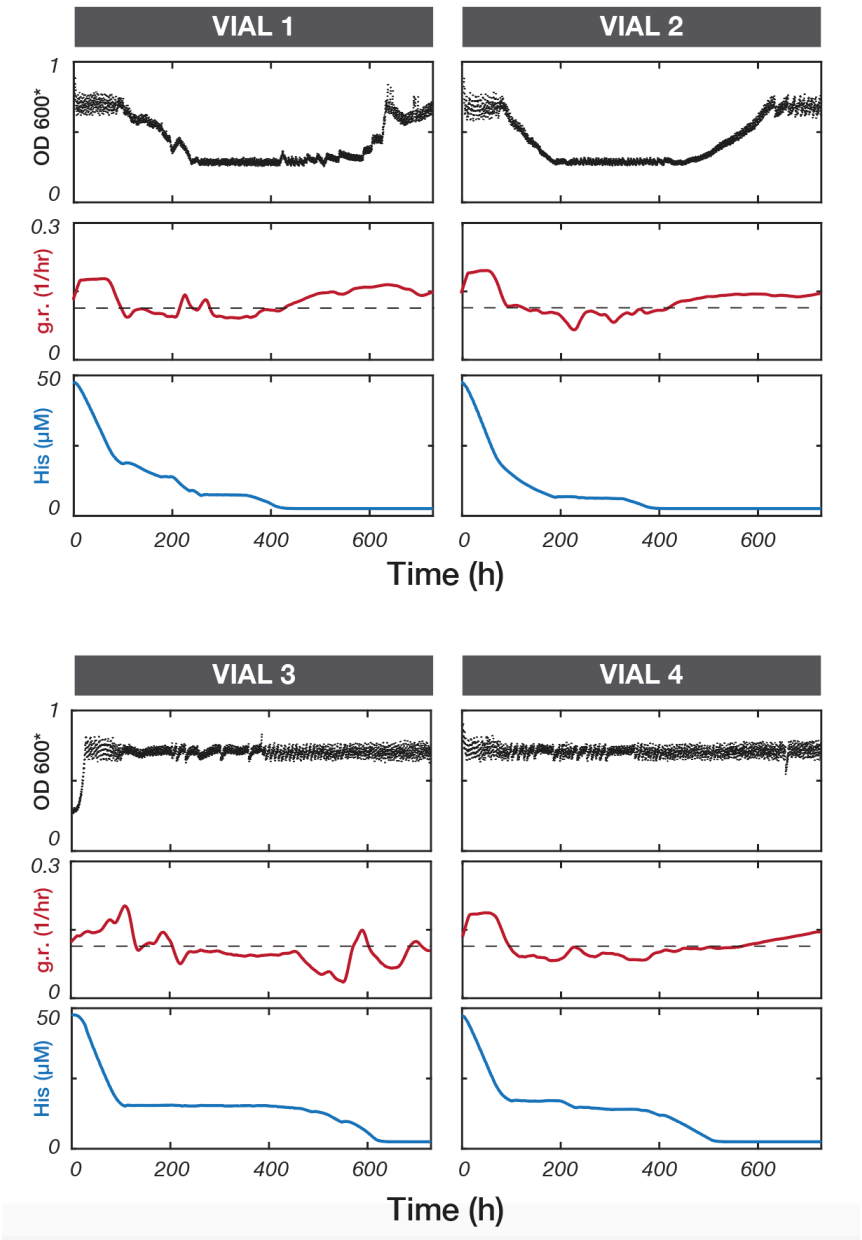

Figure S4. Adaptation history for all four replicates during *Tm*HisA evolution. OD (black dot), growth rate (red line), and histidine concentration (blue line) are plotted for all six independent replicates. All four cultures were able to adapt to media lacking histidine without the need for evolution schedules or user intervention except to provide eVOLVER with fresh base and full adaptation media in 700 hours of growth.

Table S1. *Tm*HisA mutants characterized. Both nonsynonymous mutations (**bold**) and synonymous are noted. The order of these variants (top to bottom) corresponds to the order of the variants (left to right) in Figure 3c.

Name	Mutations from wt *Tm*HisA
V1-1	**E37G**, N62N, **E71G**, **L85F**, R98R, **F124L**, K168K, **K182R**, E186E, S202S, **V209A**, **F226S**
V1-3	**G12E**, **E29K**, **V36M**, **H75Y**, **I185T**, **R224K**
V1-4	F27F, **I40T**, G43G, **E71G**, **F72L**, L112L, V123V, D127D, **A140T**, E186E, **V209A**
V1-6	**E37G**, **E71G**, **F124L**, L126L, E186E, **S199P**, **V209A**
V2-1	**A15V**, **E37G**, **E71R**, D108D, **F124L**, L172L, E186E, **V209A**
V2-2	**V4A**, **A15V**, **Y28H**, **E37G**, **E71G**, **S102G**, **A134V**, V147V, **D176Y**, **K182E**, **I183T**, E186E, **V209A**, T213T, **E228K**
V2-4	**I18T**, **E71G**, **K181R**, **V209A**, **I221V**
V3-1	**H2Q**, A6A, **E71G**, **F72L**, V123V, **V133A**, E186E, E188E, **V209A**, **E234G**
V3-2	**H2Q**, A6A, D8D, **N24S**, **E71G**, **F72L**, V123V, **V133A**, E186E, E188E, **V209A**, **E234G**
V3-3	**H2Q**, A6A, V14V, **E71G**, **F72L**, V123V, **V133A**, **I161V**, E186E, E188E, **V209A**, **E234G**
V3-5	**H2Q**, A6A, **E71G**, **F72L**, **I82V**, V123V, **V133A**, **G170D**, E186E, E188E, **V209A**, **E234G**
V4-1	**V4A**, **F27S**, **V36M**, E41E, R97R, S125S, **M236T**
V4-2	**E71G**, **F72L**, **V105A**, V123V, **I144T**, E186E, **S199A**, **V209A**, **G223R**
V4-3	D8D, **V14I**, **E37G**, **E71G**, **F135L**, **S148P**, L149L, E186E, **L203S**, **K208R**, **V209A**, **F226L**
V4-4	**V4A**, **N24S**, **V36M**, G60G, R97R, **E107G**, **F124Y**, **I166T**, **I221V**, **M236T**
V4-5	**H2Q**, **I7V**, **A15V**, K22K, E29E, **V36M**, I47I, E67E, **F124L**, **E143G**, **D145N**, **V147A**, K181K, E188E

Table S2. List of plasmids used in this study. For plasmids ZZ-Ec903 to ZZ-Ec918, see Table S1 for specific sequences.

Name	Source	Parent Plasmid	Origin of replication (yeast, bacterial)	Selection Marker (yeast, bacterial)	Notes
GA-DNA-*flo1URA3*	This work	n/a	n/a	URA3, n/a	Used to delete flo1 in strains in order to prevent flocculation
GA-Ec51	Ravikumar et al. 2018[2]	See previous work	CEN6/ARS4, ColE1	URA3, AmpR	
GA-Ec64	Ravikumar et al. 2018[2]	See previous work	n/a, ColE1	TRP1, AmpR	Recombination cassette that integrates TRP1, mKate2, *Pf*DHFR, and leu2 (538C>T) in place of wt TP-DNAP1 on the orthogonal plasmid (pGKL1)
AR-Ec633	Ravikumar et al. 2018[2]	See previous work	CEN6/ARS4, ColE1	HIS3, KanR	REV1 promoter > TP-DNAP1 L477V, L640Y, I777K, W814N
ZZ-Ec482	This work	AJ-Ec200[27]	2µ, ColE1	NatMX, AmpR	contains sgRNA targetting *TRP1* (*GTCCATTGGTGAAAGTTTG*)
ZZ-Ec475	This work	GA-Ec64	n/a, ColE1	URA3, AmpR	p1 recombination cassette that integrates *Tm*HisA and URA3 in place of ORFs 1-4 on the orthogonal plasmid (pGKL1)
ZZ-Ec506	This work	AR-Ec633	CEN6/ARS4, ColE1	LEU2, KanR	Plasmid encoding the error-prone orthogonal DNAP (TP-DNAP1 (L477V, L640Y, I777K, W814N)) driven by the REV1 promoter
ZZ-Ec727	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Sc*His6
ZZ-Ec903	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA-V1-1
ZZ-Ec904	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V1-3
ZZ-Ec905	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V1-4
ZZ-Ec906	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V1-6
ZZ-Ec907	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V2-1
ZZ-Ec908	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V2-2
ZZ-Ec909	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V2-4
ZZ-Ec910	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V3-1
ZZ-Ec911	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V3-2
ZZ-Ec912	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V3-3
ZZ-Ec913	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V3-5

ZZ-Ec914	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V4-1
ZZ-Ec915	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V4-2
ZZ-Ec916	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V4-3
ZZ-Ec917	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V4-4
ZZ-Ec918	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > *Tm*HisA -V4-5
ZZ-Ec919	This work	ZZ-Ec506	CEN6/ARS4, ColE1	LEU2, KanR	Encodes RPL18B promoter > wt *Tm*HisA

Table S3. List of yeast strains used in this study.

Name	Genotype	Source	Parent Strain	Notes
F102-2u	F102-2u *MATa can1 his4-519 leu2-3,112* ρ^0+ pGKL1 + pGKL2	ATCC #200585	n/a	
AR-Y383	F102-2u *MATa can1 his3 leu2Δ0 ura3Δ0 trp1 HIS4* ρ^0+ pGKL1 + pGKL2	Ravikumar et al., 2018[2]	AR-Y292	
ZZ-Y454	F102-2u *MATa flo1::URA3 can1 his3 leu2Δ0 ura3Δ0 trp1 HIS4* ρ^0+ pGKL1 + pGKL2	This work	AR-Y383	AR-Y383 transformed with GA-DNA-*flo1URA3*
ZZ-Y455	F102-2u *MATa flo1 can1 his3 leu2Δ0 ura3Δ0 trp1 HIS4* ρ^0+ pGKL1 + pGKL2	This work	ZZ-Y454	ZZ-Y454 streaked on solid media containing 5-FOA and confirmed with a negative flocculation phenotype
GA-Y235	F102-2u *MATa flo1 can1 his3 leu2Δ0 ura3Δ0 trp1 HIS4 dfr1::KanMX* ρ^0+ pGKL1 + pGKL2+GA-Ec51	This work	ZZ-Y455	ZZ-Y455 transformed with GA-Ec51
ZZ-Y431	F102-2u *MATa flo1 can1 his3 leu2Δ0 ura3Δ0 trp1 HIS4 dfr1::KanMX* ρ^0+ p1- FulldelPol-W-mK-PfDHFR-l*(TAA) + pGKL1 + pGKL2+GA-Ec51	This work	GA-Y235	GA-Y235 transformed with GA-Ec64
ZZ-Y435	F102-2u *MATa flo1 can1 his3 leu2Δ0 ura3Δ0 trp1 HIS4 dfr1::KanMX* ρ^0+ p1- FulldelPol-W-mK-PfDHFR-l*(TAA) + pGKL1 + pGKL2 + AR-Ec633	This work	ZZ-Y431	GA-Y235 transformed with AR-Ec633
ZZ-Y292	F102-2u *MATa can1 leu2Δ0 ura3Δ0 his6::KanMX HIS4* ρ^0+ pGKL1 + pGKL2	This work	AR-Y288	AR-Y288 transformed with PCR product from Yeast Knockout Collection strain with *his6* deleted with primers TCATCATCAAGGGTCATCTTTTTAT and GAAAAAGGTTGCCTCAATATTGTTA

ZZ-Y299	F102-2u *MATa can1 leu2Δ0 ura3Δ0 trp1Δ0 his6::KanMX HIS4* ρ^0+ pGKL1 + pGKL2	This work	ZZ-Y292	ZZ-Y292 transformed with ZZ-Ec482 and linear DNA corresponding to 40 basepairs upstream and downstream of *TRP1*. The strain was restreaked x2 on YPD solid media to remove ZZ-Ec482
ZZ-YT17-A3	F102-2u *MATa can1 leu2Δ0 ura3Δ0 trp1Δ0 his6::KanMX HIS4* ρ^0+ p1-FullDelPol-*Tm*HisA-URA3 + pGKL2 + ZZ-Ec506	This work	ZZ-Y299	ZZ-Y299 transformed with ZZ-Ec506 and ScaI-digested ZZ-Ec475
ZZ-Y323	F102-2u *MATa can1 leu2Δ0 ura3Δ0 trp1Δ0 his6::KanMX HIS4 flo1::NatMX* ρ^0+ p1-FullDelPol-*Tm*HisA-URA3 + pGKL2 + pZZ-Ec506	This work	ZZ-YT17-A3	ZZ-Y299 transformed with linear DNA containing 500 basepairs upstream of *FLO1*, *NatMX*, and 500 basepairs downstream of *FLO1*
BY4741	*MAT***a** *his3Δ1 leu2Δ0 met15Δ0 ura3Δ0* ρ^+	ATCC #201388		
ZZ-Y332	BY4741 *MAT***a** *HIS3 leu2Δ0 met15Δ0 ura3Δ0* ρ^+	This work	BY4741	BY4741 transformed with HIS3 PCR from F102-2u
ZZ-Y336	BY4741 *MAT***a** *HIS3 leu2Δ0 met15Δ0 ura3Δ0 his6::KanMX* ρ^+	This work	ZZ-Y332	ZZ-Y332 transformed with PCR product from Yeast Knockout Collection strain with *his6* deleted with primers TCATCATCAAGGGTCATCTTTTTAT and GAAAAAGGTTGCCTCAATATTGTTA
ZZ-Y354	BY4741 *MAT***a** *HIS3 leu2Δ0 met15Δ0 ura3Δ0 trp1Δ0 his6::KanMX* ρ^+	This work	ZZ-Y336	ZZ-Y292 transformed with ZZ-Ec482 and linear DNA corresponding to 40 basepairs upstream and downstream of *TRP1*. The strain was restreaked x2 on YPD solid media to remove ZZ-Ec482

References

(27) Javanpour, A. A., Liu, C. C. (2019) Genetic Compatibility and Extensibility of Orthogonal Replication. *ACS Synth. Biol., 8*, 1249–1256.