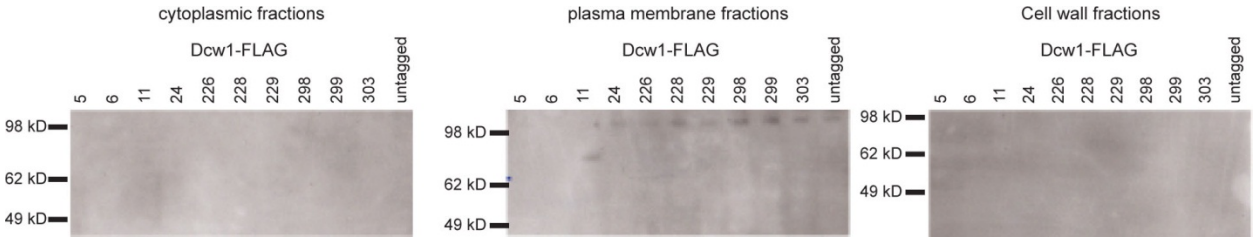

Figure S1. Western blot analysis of Dcw1-FLAG strains with α-DCW1 antibody

The same cell fractions used in Figure 5 were run on new SDS-PAGE gels and probed with an α-Dcw1 antibody. Signal was detected using chemiluminescence, and film was exposed for 45 minutes. Material from $7.5x10^5$ cells is loaded in each strain in each fraction. Wells are labeled with the amino acid site of the FLAG tag within Dcw1. We note the apparent size of Dcw1 in the plasma membrane is larger than what we observed with α-FLAG antibodies, but the overall pattern is consistent with the α-FLAG Westerns shown in Figure 5.

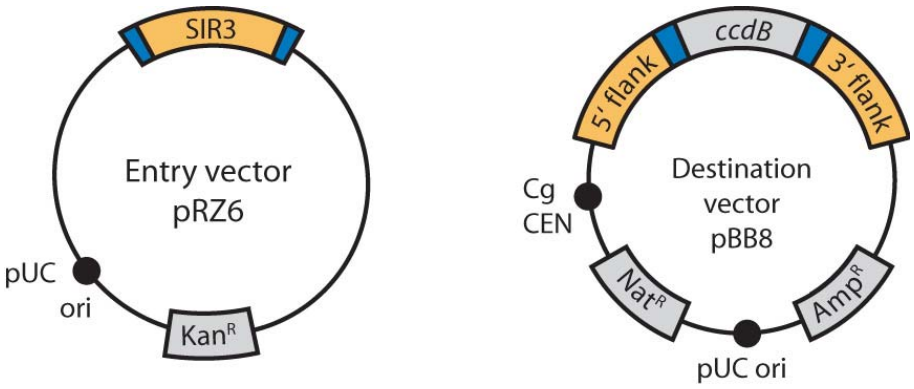

Figure S2. Gateway constructs used for mutagenesis of *C. glabrata SIR3*
These schematic drawings represent the Gateway entry vector and destination vector used during the mutagenesis of *C. glabrata SIR3*, as represented in Figure 2 in the manuscript.

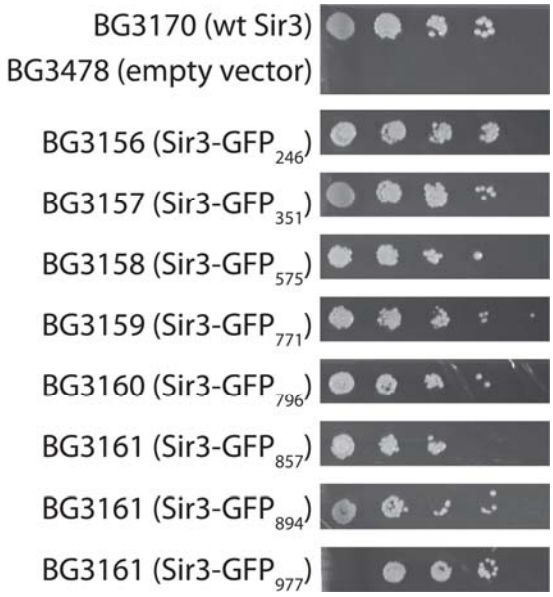

Figure S3. Sir3-GFP alleles complement for growth on 5-FOA

C. glabrata strains carrying various Sir3-GFP alleles were grown overnight in YPD+Nat media in 96-well plates. Yeast were diluted to OD_{600}=2 and placed in the left-most well of a new 96-well plate. Ten-fold serial dilutions were made in sterile PBS, and the cells were transferred to a 5-FOA plate using a multi-pin tool. The plate was grown at 30°C. We note that the lack of growth for BG3161 in the left-most (densest) dilution is likely due to uneven placement of the pin tool onto the 5-FOA plate.

Table S1. Plasmids used in this study

Plasmid	Description	*E. coli* marker	Yeast marker
Tn7 donor plasmids			
pRZ49	Tn7-mEOS2 donor vector	Kan	n/a
pRZ98	Tn7-biotin-donor vector	Kan	n/a
pRZ99	Tn7-6His donor vector	Kan	n/a
pRZ101	Tn7-FLAG donor vector	Kan	n/a
pRZ102	Tn7-HA donor vector	Kan	n/a
pRZ103	Tn7-myc donor vector	Kan	n/a
pRZ106	Tn7-GFP donor vector	Kan	n/a
DCW1 plasmids			
-	pCU-*DCW1*	Amp	*URA3*
-	*DCW1* entry vector	Kan	n/a
pRZ159	empty *DCW1* destination vector	Amp	*HIS1*
pRZ160	wild type *DCW1* expression vector	Amp	*HIS1*
pRZ165	Dcw1-$FLAG_5$	Amp	*HIS1*
pRZ166	Dcw1-$FLAG_6$	Amp	*HIS1*
pRZ172	Dcw1-$FLAG_{11}$	Amp	*HIS1*
pRZ173	Dcw1-$FLAG_{24}$	Amp	*HIS1*
pRZ174	Dcw1-$FLAG_{226}$	Amp	*HIS1*
pRZ175	Dcw1-$FLAG_{228}$	Amp	*HIS1*
pRZ167	Dcw1-$FLAG_{229}$	Amp	*HIS1*
pRZ168	Dcw1-$FLAG_{298}$	Amp	*HIS1*
pRZ176	Dcw1-$FLAG_{299}$	Amp	*HIS1*
pRZ177	Dcw1-$FLAG_{303}$	Amp	*HIS1*
pBC715	Dcw1-HA_{26}	Amp	*HIS1*
SIR3 plasmids			
pRZ6	*SIR3* entry vector	Kan	n/a
pBB8	empty *SIR3* destination vector	Amp	Nat^R
pRZ47	wild type *SIR3* expression vector	Amp	Nat^R
pRZ120	Sir3-GFP_{246}	Amp	Nat^R
pRZ121	Sir3-GFP_{351}	Amp	Nat^R
pRZ122	Sir3-GFP_{575}	Amp	Nat^R
pRZ124	Sir3-GFP_{771}	Amp	Nat^R
pRZ127	Sir3-GFP_{796}	Amp	Nat^R
pRZ130	Sir3-GFP_{857}	Amp	Nat^R
pRZ131	Sir3-GFP_{894}	Amp	Nat^R
pRZ133	Sir3-GFP_{977}	Amp	Nat^R

Table S2. Primers used in this study

Oligo #	Name	Sequence (5'-3')
Tn7-tag donor vector construction		
3201	Tn7L-FseI-Bam-for	aggactacggatcctgtggccggccAATAAAGTCTTAAACTGAACAAA
3202	Tn7L-AscI-rev	gacctgacggcgcgccGTCGACCCCACGCCCCTCTTTAAT
4724	mEOS_for	aggcgcgccggccggccTGGATCCGCTGGCTCCGCTGCTGGTTCTGGC GAATTCATGAGT
4725	mEOS_rev	gctctagagtttaaacTAAATTCTCCAGATCCTGCAGCAGATCCTGCAGAGCCTCGTCT GGCATTGTCAGGCAATCCAGAATGAG
5300	L1-GFP-f	atttaggatccgctggctccgctgctggttctggcATGTCTAAAGGTGAAGAATTATTCACTGG TG
5301	L2-GFP-r	ataagtttaaactagctcctcctgcagcagatcctgcagagccTTTGTACAATTCATCCATACCAT GGGTAATAC
DCW1-Yiplac211 knockout construct		
1975	*DCW1* 3'flank rev SphI	acatgcatgcAGGAAACCATGTAAGCGATGAATAT
1976	*DCW1* 3'flank for KpnI	ggggtaccTGCAGAACTTATGAAAGCTTAACATTT
1977	*DCW1* 5'flank rev KpnI	ggggtaccTTTTATGTGTTCGTTTTTAAAACAGAC
1978	*DCW1* 5'flank for HindIII	ccccaagcttAGATGAACTTGAACTTAAGATGATC
Amplify across *DCW1* knockout region		
2306	*DCW1* 5' check	TCGTTTAAATTCAATTGGAACTGTA
2307	*DCW1* 3' check	TTCAAACAAAATTCGTTCGATATTA
Verify *DCW1*-Yiplac211 integrants		
1504	Yiplac backbone	TATGTTGTGTGGAATTGTGAGCGG
1505	URA check	GCGATTAAGTTGGGTAACGCCAGG
Verify loopout of *DCW1*-Yiplac211 construct		
1778	*DCW1* 5' check	ACCTTTCCAGGACATATAAT
1779	*DCW1* 3' check	ACACATATGAACAAAGGTCT
pCU-*DCW1* construction		
1629	3ecoYKL046	ccggaattcTCAAAAGACTAACCACAGAGCACATG
1630	5bamYKL046	cgcgggatccATGCTAGTAAATAAAGTGATAGGGT
DCW1 destination vector		
6333	*DCW1* promoter - for	atagagctcTTCTTCTCCTTATTGTGCTTTACC
6334	*DCW1* promoter - rev	attctagaTTTTATGTGTTCGTTTTTAAAACAGACTG
Determine position of FLAG insertion.		
2766	*DCW1* promoter - for	GATGATCATAGGTACTCTTTGTATAATGGGC
6244	linker 2 - rev	ATTAGTTTAAACTAGCTCCTCCTGCA
5160	linker 1 - for	CTCCGCTGCTGGTTCTGG
4032	M13F (-21)	GTAAAACGACGGCCAGT
5626	Tn7L - rev	GATCTATTTTGTTCAGTTTAAGACTTTATTG
Sir3 entry vector construction		
4470	SIR3b1	ggggacaagtttgtacaaaaaagcaggctaaaaccATGGCTGAGCTTATAAAAGACCTG
4484	SIR3b2	ggggaccactttgtacaagaaagctgggtCTATTCGGTGAGACACGATTGGAT

Sir3 destination vector construction			
	3220	Sir3_IP_SacI_F	gtacctatgagctcGAACGGTGCCAGACACACCAGCCC
	3221	Sir3_IP_XbaI_R	tgaccatatctagaCCTCTTACTTAATCCGAAACCTTC
	3222	Sir3_UTR_XhoI_F	caatgcacactcgagAAAAGCTTTCATCTTCTTTTCTTGATTCTCCTC
	3223	Sir3_UTR_KpnI_R	catgaccatggtaccAAGACGGCTCCATCACTAAAGTGC
Determine position of epitope insertions within *SIR3* using colony PCR and sequencing			
	5160	L1 for	CTCCGCTGCTGGTTCTGG
	5161	L2 rev	CTCCTCCTGCAGCAGATCCT
	5602	SIR3IP_3'for	CTGGGAAGGTTTCGGATTAAGTAAGAGG
	5605	SIR3utr_5'rev	GTATTAGTAGAGGAGAATCAAGAAAAGAAGATGAAAG
	5627	Linker2+myc	GATCCTGCAGAGCCTTCATTGAG
	5738	Bio_L2-rev	GATCCTGCAGAGCCTTCATGCC
	5739	GFP 5' rev	AGGTCAATTTACCGTAAGTAGCATCAC
	5740	GFP 3' for	TTATCCACTCAATCTGCCTTATCCA
	5741	mEOS 5' rev	CGAATACCCTGTTGCCGTAATGGA
	5742	mEOS 3' for	ACCGATGTGACTTCAGAACTACTTACAAAG

Bases that anneal to the template are shown in capital letters; restriction sites, linkers, and Gateway recombination sites added to the primers are shown in lowercase letters.

Table S3. *S. cerevisiae* strains used in this study

Strain	Genotype	Parent	Source
BY4741	MAT**a** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0		[Brachmann, 1998 #76]
BY4742	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0		[Brachmann, 1998 #76]
*dfg5*Δ	MAT**a** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *dfg5*::Kan^R	BY4741	[Winzeler, 1999 #79]
*dcw1*Δ	MAT**a** *his3*Δ1 *leu2*Δ0 *met15*Δ0 *ura3*Δ0 *dcw1*(YKL046C)::Kan^R	BY4741	[Winzeler, 1999 #79]
BY240	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pCU-*DCW1*	BY4742	this work
BY965	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pCU-*DCW1* pRZ159	BY240	this work
BY966	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ160	BY240	this work
BY893	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ165	BY240	this work
BY895	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ166	BY240	this work
BY974	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ172	BY240	this work
BY975	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ173	BY240	this work
BY976	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ174	BY240	this work
BY977	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ175	BY240	this work
BY897	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ167	BY240	this work
BY899	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ168	BY240	this work
BY978	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ176	BY240	this work
BY979	MATα *his3*Δ1 *leu2*Δ0 *lys2*Δ0 *ura3*Δ0 *dfg5*::g418 *dcw1*Δ pRZ177	BY240	this work

Table S4. *C. glabrata* strains used in this study

Strain	Genotype	Parent	Source
CGM293	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4*	CGM693	[Rosas-Hernandez, 2008 #105]
BG3156	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ120	CGM293	this work
BG3157	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ121	CGM293	this work
BG3158	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ122	CGM293	this work
BG3159	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ124	CGM293	this work
BG3160	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ127	CGM293	this work
BG3161	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ130	CGM293	this work
BG3162	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ131	CGM293	this work
BG3163	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ133	CGM293	this work
BG3170	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pRZ47	CGM293	this work
BG3478	*ura3Δ*::Tn*903* G418^R, *sir3Δ::hph* (pIC120/*Kpn* I-*Hpa* I+T4 DNA polymerase). *URA3* at unique region between *EPA5* and *EPA4* pBB8	CGM293	this work

Table S5. **Drug resistance of isolates from xpC culture, as assessed from replica plating.**

Source plate	$Tmp^RKan^RCar^R$ colonies	Tmp^RCar^R colonies	Kan^RCar^R colonies	Only Car^R colonies	Total Car^R colonies	Tmp^RCar^R/ (total Car^R) correction
1	2	14	18	150	184	7.6%
2	5	9	24	119	157	5.7%
3	2	5	24	166	197	2.5%
4	1	8	23	158	190	4.2%

"Total Car^R colonies" is the sum of all colonies on the previous 4 columns (and matches the total number of colonies on the original LB+Car source plate. Tmp^RCar^R /(total Car^R) correction indicates the percentage of all Car^R colonies that are in fact Tmp^R Car^R. This represents the % of the xpC pool that bears a mutagenized *DCW1*-FLAG expression vector, and was used to correct the xpC pool size shown in Table 1 of the main paper.

Table S6. Size of *SIR3* mutagenesis pools during selection and sequencing

Colony counts, as plated after recovery during transformation, prior to drug selection in pool. Cells were diluted appropriately to prevent a lawn of growth and plated onto media as indicated. Numbers in the table are calculated to represent the number of colonies in the full transformation resistant to the given drug. nd = not determined. Fold coverage was calculated by comparing the number of colonies from the indicated drug selection to the size of the DNA available for mutagenesis (Supplemental Note 1). * The Sir3-myc pool was treated differently than other pools. After transformation of the Sir3-myc pool into *C. glabrata*, the pooled culture was outgrown in YPD+NAT for ~24 hours prior to plating on YPD+NAT. All other tagged Sir3 pools were plated directly onto YPD+NAT plates following transformation and recovery.

		Tn used to mutagenize *C. glabrata SIR3*							
Step of processing	**Fold coverage based on which drugs?**	**myc**		**bio**		**mEOS2**		**GFP**	
		# colonies	**fold coverage**	**# colonies**	**fold coverage**	**# colonies**	**fold coverage**	**# colonies**	**fold coverage**
Mutagenesis of entry vector	Tmp Kan	5.4E+05	67x	2.9E+05	36x	3.2E+05	39x	6.46E+05	**79.99**
xpC = Gateway LR to create expression pool	Tmp Car	6.0E+06	920x	6.0E+06	920x	6.0E+06	920x	6.00E+06	920x
xpT	Tmp Car	5.7E+07	8700x	8.2E+07	13000x	1.8E+07	2800x	2.70E+07	4100x
xpT-Tn7L	Tmp Car	1.2E+07	1800x	3.6E+07	5500x	4.1E+07	6200x	2.34E+07	3600x
xpT-Tn7L-Tn7R	Car	1.6E+07	2400x	5.8E+07	8900x	6.2E+07	9500x	3.50E+07	5400x
Transformation into *C. glabrata*	Nat	7992*	1.2x	2172	0.33x	2432	0.37x	1895	0.29x
Screening for functional isolates	5-FOA	906	n/a	190	n/a	162	n/a	105	n/a
% functional clones		11%	n/a	8.7%	n/a	6.7%	n/a	5.5%	n/a
Sent for sequencing		95		63		52		48	
# mappable sequences		90		53		47		48	
# isolates with inframe insertions in SIR3 orf		88		52		46		47	
# in-frame unique insertions sites		21		42		33		30	
Max # insertions at a single site		27		4		6		6	

Insertion site (amino acid)	Epitope tag			
	myc	bio	mEOS2	GFP
158		1		
171			1	
246				5
257		1		
263		1		
275		1		
277	1			
278		1		
279		1		
289		1		
294		1		
338		1		
351		1		2
352		1		
370		4		
380	1	1		
393	3			
395	15			
396		1		
399		1		
405		1		
408				1
413	3			
414		2		
458	5			
475				1
507		2		
536	1			2
543			1	
544	1			
546		1		
548		1		
550			1	
551			1	
552			2	
553				1
573	2			1
574	1			
575				1
590			2	
629				1
645		1		
667		1	1	
668		1		
669			1	
712		1	1	
713			1	
716	2	1	1	
717		1	1	
721	1			1
749			1	
767				1

Table S7. Insertion sites of select in-frame complementing tagged Sir3-alleles

Complementing clones from the mutagenized Sir3 pools were analyzed with colony PCR to qualitatively determine the location of the epitope tag within the *SIR3* ORF. PCR products from a subset of these complementing clones were sequenced and the precise location of the tag insertion was identified. Each of the 107 unique locations within Sir3 where a tag was found in a functional allele is listed in the "insertion site" column, based on the amino acid of insertion. The final two insertion sites listed are downstream of the Sir3 STOP codon. The remaining columns list the number of complementing clones from each mutagenized pool found at a given insertion location. In total, the epitope tag was located in frame in 65 Sir3-myc, 52 Sir3-bio, 46 Sir3-mEOS2, and 47 Sir3-GFP complementing clones.

Insertion Site (Amino acid)	Epitope tag			
	myc	bio	mEOS2	GFP
769		1	4	
771				1
772			1	
773			1	
775				1
777		2		
793				1
795	2			
796		3	2	4
797				1
800		1		
804			1	
807	7	3	6	6
808				1
809	2			
826	27			
829			1	
857			1	1
858			2	
859			1	
860			1	
863	1		1	
870			1	
877				1
878			1	
887				1
889		1		
894			2	1
895	2		1	1
896				1
918			1	
922				1
931				1
972				1
975	7		1	1
976		1		
977				4
981	3			
984		1		
997		1	1	
998		1		
1002	1			
1012		1		
1013		1		
1015		1		
1016				1
1017		1		
1019		1		
1081			1	
1084		1		
_+6 bp	1			
+17 bp	1			

Table S7 (continued). Insertion sites of select in-frame complementing tagged Sir3-alleles

Complementing clones from the mutagenized Sir3 pools were analyzed with colony PCR to qualitatively determine the location of the epitope tag within the *SIR3* ORF. PCR products from a subset of these complementing clones were sequenced and the precise location of the tag insertion was identified. Each of the 107 unique locations within Sir3 where a tag was found in a functional allele is listed in the "insertion site" column, based on the amino acid of insertion. The final two insertion sites listed are downstream of the Sir3 STOP codon. The remaining columns list the number of complementing clones from each mutagenized pool found at a given insertion location. In total, the epitope tag was located in frame in 95 Sir3-myc, 52 Sir3-bio, 46 Sir3-mEOS2, and 47 Sir3-GFP complementing clones.

File S1

Protocol

In vitro Transposon Mutagenesis for Introduction of Internal Epitope Tags

REQUIRED REAGENTS

Purified TnsA, TnsB, and TnsC^{A255V} enzymes [3, 4]

TnsA stock = 150ng/μl in Storage Buffer A

TnsB stock = 200ng/μl in Storage Buffer B

TnsC ^{A255V} stock = 500ng/μl in Storage buffer C

Tn7 donor vector: pRZ101 (Tn7-FLAG donor vector). Dilute to 25ng/μl in 10mM Tris pH8.0

Entry vector of target gene: *DCW1* entry vector. Dilute to 50ng/μl in 10mM Tris pH8.0

Invitrogen MegaX DH10B T1R electrocompetent *E. coli* cells

300mM Magnesium Acetate (MgOAc)

phenol:chloroform:IAA (25:24:1) (Amresco 0883-100ml)

Chloroform

3M Sodium Acetate (NaOAc)

100% ethanol (EtOH)

70% EtOH

FseI (New England Biolabs R0588S)

PmeI (New England Biolabs R0560S)

ApaLI (New England Biolabs R0507S)

T4 DNA ligase and buffer (New England Biolabs M0202S)

Gateway LR clonase II (Life Techonologies 11791-100)

Qiagen Hi-Speed MidiPrep kit (Qiagen 12643)

MegaX DH10B T1R Electrocomptent Cells (Life Technologies C6400-03)

LB media

Oxoid Isosensitest media (Iso) (agar: OXCM0471B, liquid broth: OXCM0473B)

Carbenicillin (100mg/ml = 1000x stock)

Kanamycin (30mg/ml = 1000x stock)

Trimethoprim (5mg/ml in DMSO = 500x stock)

ddH_2O = doubly distilled (MilliQ) H_2O

BUFFERS

Use sterile-filtered MilliQ water to make all buffers. If possible, make all buffers in plastic containers; residual detergent on glassware may inhibit the transposition reaction.

Standard buffers

20mM ATP in 125mM Tris pH 7.5 (store at -20°C for at most 1 month)
20mM DTT in 125mM Tris pH 7.5 (store at -20°C for at most 1 month)
100mM ATP in 250mM HEPES (pH8.0) – for making TnsC storage buffer
1M DTT in 150mM HEPES (pH8.0) – for making TnsA and TnsC storage buffers
1M DTT in 1M Tris pH 7.5 – for making TnsB storage buffer
10mM Tris - for elution of mutagenized plasmid pools from midiprep kit
50% glycerol

Tns storage buffers

Store at -20°C for at most 6 months. We do not recommend refreezing and rethawing of the storage buffers, so store them in small aliquots and discard after use.

Storage buffer A
25mM HEPES pH8.0
150mM NaCl
1mM EDTA
1mM DTT (in HEPES)
10% glycerol

Storage buffer B
25mM TrisHCl pH8.0
500mM NaCl
1mM EDTA
1mM DTT (in Tris)
25% glycerol

Storage buffer C
25mM HEPES pH8.0
1M NaCl
0.1mM EDTA
2.5mM DTT (in HEPES)
1mM ATP
10mM $MgCl_2$
10% glycerol

***In vitro* transposition protocol**

This protocol delineates the steps to perform a small (20μl) "- enzyme" negative control reaction and a large (80μl) "+ enzyme" experimental mutagenesis reaction in parallel. The enzymes and buffers are mixed together in a master mix (5.5-reaction sized, to account for pipetting error) and later split into appropriate reaction sizes.

1) Make reaction mix. Combine:
   - 17.6 μl target DNA (880 ng) (*DCW1* entry vector)
   - 8.8 μl Tn7 donor DNA (220 ng) (pRZ101)
   - 11 μl 20mM ATP
   - 11 μl 20mM DTT
   - 56.1 μl ddH_2O

2) Aliquot reaction mix into two PCR tubes. Dispense 76μl into the "+ enzyme" reaction tube, and 19μl into the "- enzyme" reaction tube.

3) Make the enzyme mixture. Combine:
   - 7.49 μl TnsA
   - 3.31 μl Storage buffer A
   - 2.00 μl TnsB
   - 6μl Storage buffer B
   - 8μl $TnsC^{A255V}$
   - 8μl Storage buffer C
   - 5.2μl 50% glycerol

   Mix by flicking tube gently. Keep on ice while setting up transposition reaction.

   Note 1: The ratio of TnsA, TnsB, and $TnsC^{A255V}$ in this mixture was determined empirically. You may need to optimize the relative amount of each enzyme using your purified enzyme stocks.

   Note 2: You may refreeze the enzyme mixture at -80°C and rethaw twice, but will have decreased transposition efficiency with each thaw. We do not recommend refreezeing the stock solution of the individual Tns enzymes, so take care to store these in small aliquots so as to avoid wasting purified enzyme.

4) Make the buffer mixture for the "- enzyme" control. Combine:
   - 10.8 μl Storage buffer A
   - 8.0 μl Storage buffer B
   - 16.0 μl Storage buffer C
   - 5.2 μl 50% glycerol

5) Add 4μl enzyme mix to the "+ enzyme" tube. Flick tube to mix.
6) Add 1μl of the buffer mixture to the "- enzyme" tube. Flick tube to mix.
7) Incubate both tubes at 37°C for 10 minutes on a PCR heat block.
8) Add 300mM MgOAc to the tubes:
   For "+ enzyme" reactions, add 4.2μl 300mM MgOAc
   For "- enzyme" reaction, add 1.05μl 300mM MgOac
9) Incubate at 37°C for 1 hour on a PCR block
10) Incubate at 75°C for 5 minutes on a PCR block to heat-kill the enzymes.

Clean-up of transposition reactions

11) Transfer "+ enzyme" and "- enzyme" reactions to 1.5ml microfuge tubes. Bring the volume of each up to 100µl.
12) Add 100µl phenol:chloroform:IAA. Vortex to mix.
13) Spin 5 minutes, 4°C, 13500 rpm in a microfuge. Remove and discard organic (bottom) layer.
14) Add 100µl chloroform. Vortex to mix.
15) Spin 5 minutes, 4°C, 13500rpm in microfuge. Transfer aqueous (top) later to a new 1.5ml microfuge tube.
16) Add 10µl 3M NaOAc. Add 220µl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
17) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
18) Wash DNA pellet with 500µl ice-cold 70% EtOH.
19) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
20) Resuspend DNA pellets in desired volume of 10mM Tris pH 7.5.
    For "+ enzyme" reactions, use 8 µl Tris pH 7.5
    For "- enzyme" reaction, use 4 µl Tris pH 7.5
21) Optional: Cleaned transposition reactions may be stored at -20°C prior to transformation.

Transformation of transposition reactions

Transform cleaned transposition reaction DNA into Invitrogen MegaX DH10B T1R electrocompetent *E. coli* cells. One set of steps is described for the "+ enzyme" reactions, another for "- enzyme" reactions. They can be performed in parallel, but are separated here for clarity.

For "- enzyme" reactions:

22A) Combine 2μl DNA with 20μl MegaX cells in a chilled electroporation cuvette.
23A) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24A) Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.
25A) Transfer 900μl cells to a 1.5ml microfuge tube.
26A) Recover at 37°C for 1 hour.
27A) Plate 9μl (1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan .
28A) Grow plates overnight at 37°C.

For "+ enzyme" reactions:

22B) Combine 4μl DNA with 40μl MegaX cells in one chilled electroporation cuvette.
23B) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24B) Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.
25B) Transfer 900μl cells to a 500ml flask containing 150 mls Isosensistest media.
26B) Recover at 37°C for 1 hour
27B) Plate 150μl (0.1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan
28B) Add 150μl of Kan (30mg/ml stock) and 300μl Tmp (5mg/ml stock) to the flask.
29B) Grow plates and culture overnight at 37°C.

30) The following morning, count the colonies growing on each plate. Calculate the number of independent transformants in your "+ enzyme" pools.
31) Make a glycerol frozen stock of the "+ enzyme" overnight culture. Store at -80°C.
32) Pellet the remainder of the "+ enzyme" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 33 directly.
33) Purify the DNA from the "+ enzyme" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the mutagenized *DCW1**FLAG entry vector pool (ep).

Note: You can monitor the plasmid population content by restriction enzyme digestion of the DNA pool. We have observed about 50% of the plasmid pool will be unmutagenized target vector. We suspect that the MegaX may take up multiple plasmids during transformation and do not segregate them properly.

Gateway LR mobilization to create expression pools

Linearizing the mutagenized entry pool increases the efficiency of the Gateway LR reaction. Additionally, it minimizes the amount of Kan-marked plasmid that is present in the subsequent pools.

Linearize mutagenized DNA pool

1) Identify a restriction enzyme that cuts only in the backbone of the mutagenized *DCW1**FLAG pool. We chose ApaLI, which cuts in the origin of replication.
2) Digest 1μg of DNA from the *DCW1**FLAG mutagenized entry pool in a 20μl reaction. Include a negative control – use 100ng of DNA in a 20μl reaction.
3) Digest 37°C for 1 hour.
4) Run the entire negative control reaction, and 2μl of the ApaLI digestion, on a gel to verify the backbone is fully digested. If digestion was successful, proceed.

Clean the linearized DNA

5) Add 82μl ddH2O to the remaining ApaLI-digested DNA, to bring it to a final volume of 100μl.
6) Add 100μl phenol:chloroform:IAA. Vortex to mix.
7) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Remove and discard organic (bottom) layer.
8) Add 100 μl chloroform. Vortex to mix.
9) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Transfer aqueous (top) later to a new 1.5ml microfuge tube.
10) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
11) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
12) Wash DNA pellet with 500μl ice-cold 70% EtOH.
13) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
14) Resuspend DNA in 20μl 10mM Tris. DNA will be 45ng/μl.

Gateway LR Reaction – Move ORF to destination vector

1 reaction volume uses 150ng destination vector and 150ng (mutagenized, linearized) entry vector

15) Make a 2.5x reaction mixture DNA, which will be split into +LR and –LR reactions. Combine: 371.25ng (8.25μl) mutagenized, linearized entry vector
    371.25ng pRZ159 (*DCW1* destination vector)
    Bring to 20μl total volume with TE
16) For the "+LR" experimental reaction, combine 16μl of the DNA mixture from step 15 and 4μl Invitrogen LR Clonase II in a PCR tube. For the "-LR" control reaction, combine 4μl DNA mixture from step 15 and 1μl TE in a PCR tube. Incubate both reactions overnight at 25°C on the PCR block.
17) Stop the LR reaction by adding proteinase K to the reactions: Add 2μl proteinase K to the "+LR" experimental reaction; Add 0.5μl proteinase K to the "-LR" negative control.
18) Incubate at 37°C for 10 minutes on the PCR block.
19) Clean reactions: Transfer reactions to 1.5ml microfuge tubes. Perform phenol/chloroform extraction and EtOH precipitation as described before (steps 5-13 under "Clean linearized DNA")
20) Resuspend "+LR" experimental DNA in 6μl TE (final [DNA] = 50ng/ul).
21) Resuspend "-LR" negative control DNA in 4μl TE (final [DNA] = 18.75ng/ul).

Transform Gateway LR reactions

Transform cleaned LR reaction DNA into Invitrogen MegaX DH10B T1R electrocompetent *E. coli* cells. One set of steps is described for the "+ enzyme" reactions, another for "- enzyme" reactions. They can be performed in parallel, but are separated here for clarity.

Note that the Car and Tmp selections (intended to select only those expression plasmids with a DCW1 orf and a Tn insertion) are performed in sequential rounds of transformation. We found simultaneous Car and Tmp drug selection seemed to pressure cells into maintaining both an unmutagenized DCW1 expression vector and a mutagenized entry vector.

For "- enzyme" reactions:

22A) Combine 2μl DNA with 20μl MegaX cells in a chilled electroporation cuvette.
23A) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24A) Add 1ml Recovery media (provided with MegaX cells) to the cuvette and pipet up and down to resuspend.
25A) Transfer 900μl cells to a 1.5ml microfuge tube.
26A) Recover at 37°C for 1 hour.
27A) Plate 9μl (1% of total cells) onto three types of selective media – LB+Kan, Iso + Tmp, and Iso+Tmp+Kan .
28A) Grow plates overnight at 37°C.

For "+ enzyme" reactions:

22B) Combine 2μl DNA with 20μl MegaX cells in one chilled electroporation cuvette.
23B) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
24B) Add 1ml Recovery media (provided with MegaX cells) to the cuvette and pipet up and down to resuspend.
25B) Transfer 900μl cells to a 1.5ml microfuge tube.
26B) Recover at 37°C for 1 hour
27B) Transfer contents of microfuge tube to a 500ml flask containing 150mls LB. Swirl to mix.
28B) Plate 150μl (0.1% of total cells) onto five types of selective media – LB+Car, LB+Kan, Iso + Tmp, and Iso+Tmp+Kan , Iso+Tmp+Car
29B) Add 150μl of Car (100mg/ml stock) to the flask.
30B) Grow plates and culture overnight at 37°C.

30) The following morning, count the colonies growing on each plate. Calculate the number of independent transformants in the "+ enzyme" pools.
31) Make a glycerol frozen stock of the "+ enzyme" overnight culture. Store at -80°C.
32) Pellet the remainder of the "+ enzyme" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 33 directly.
33) Purify the DNA from the "+ enzyme" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool, selected for Car^R ("xpC").

Retransform for 2nd round drug selection: Select expression pool for plasmids with Tn insertions

34) Combine 1μl "xpC" expression pool DNA (50ng/μl) with 20μl MegaX cells in one chilled cuvette.
35) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
36) Add 1ml Oxoid Isosensitest media to the cuvette and pipet up and down to resuspend.
37) Transfer 900μl cells to 150mls Isosensitest media (in a 500ml flask).
38) Recover at 37°C for 1 hour
39) Plate 150μl (0.1% of total cells) onto five types of selective media – LB+Car, LB+Kan, Iso + Tmp, and Iso+Tmp+Kan , Iso+Tmp+Car
40) Make a 1:1000 dilution of recovered cells (2μl cells into 2ml Isosensitest media). Plate 150μl of a 1:1000 dilution of cells onto Iso+Tmp, LB+Car, and Iso+Tmp+Car plates
41) Add 300μl of Tmp (5mg/ml stock) to the flask.
42) Grow plates and culture overnight at 37°C.
43) The following morning count the colonies growing on each plate. Calculate the number of independent transformants in your pools.
44) Make a glycerol frozen stock of the overnight culture. Store at -80°C.
45) Pellet the remainder of the overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 46 directly.
46) Purify the DNA from the pelleted cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool, now selected for Tmp^R ("xpT").

Restriction digestions to remove Tn7 DNA from plasmids

At this point, the pools contain expression plasmids with mutagenized target ORF. This is based on the ability to grown in MegaX cells – confirming the expression backbone instead of the entry or destination vectors, and the selection for both Car^R and Tmp^R - confirming the expression backbone (Car^R) and the presence of Tn7 (Tmp^R). At this time, the Tn7 ends and Tmp^R gene are no longer needed, and are removed from the plasmids using a series of restriction digests.

Remove Tn7L end with *Fse*I digest

1) Digest 500ng of xpT pool with *Fse*I; use 100ng of xpT pool for a "no enzyme" control.

No enzyme	***Fse*I**	
100 ng	500 ng	xpT pool DNA
1 μl	1 μl	10x NEB buffer #4
1 μl	1 μl	10x BSA
0 μl	0.5 μl	*Fse*I
To 10μl total	To 10μl total	dH2O

2) Incubate restriction digests and control reactions at 37°C for 1hr.
3) Heat inactivate *Fse*I by incubating reactions at 65°C for 20 minutes.
4) Run all of the "no enzyme" control and 2μl (100ng of DNA) from *Fse*I digest on an agarose gel to confirm restriction digest was successful. The uncut control should contain supercoiled plasmid, and the *Fse*I digest should have a high MW band of linearized plasmid DNA; the Tn7L fragment is 211bp long, though this fragment is sometimes not visible. If successful, continue with protocol.

Ligate to recircularize plasmid after *Fse*I digestion

5) Add 12μl of dH2O to remaining *Fse*I-digested xpT material to bring final volume to 20μl.
6) Set up ligation reaction, and a negative control, as shown:

- control	**+ ligase**	
10 μl	10 μl	DNA (200ng)
5 μl	5 μl	10x T4 ligase buffer
5 μl	5 μl	10mM ATP
0 μl	2.5 μl	T4 DNA ligase
30 μl	27.5 μl	dH2O
50 μl	50 μl	TOTAL

7) Ligate at room temperature, 30 minutes.
8) Heat inactivate ligase enzyme by incubating at 65°C, 20 minutes

Clean the ligated DNA after removal of Tn7L

9) Add 50μl ddH2O to the ligated DNA, to bring it to a final volume of 100μl.
10) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
11) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
12) Wash DNA pellet with 500μl ice-cold 70% EtOH.
13) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
14) Resuspend DNA in 4μl 10mM Tris. DNA will be roughly 50ng/μl

Transform ligated DNA after removal of Tn7L

For both "- ligase controls" and "+ ligase" reactions:

15) Combine 1μl of DNA (at 50ng/μl) with 20μl MegaX cells in a chilled electroporation cuvette.
16) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
17) Add 1ml Isosensitest to the cuvette and pipet up and down to resuspend.
18) Transfer 900μl cells to a 1.5ml microfuge tube.
19) Recover at 37°C for 1 hour.
20) Make a 1:10 dilution of recovered cells (22μl cells + 200μl Isosensitest). Plate 90μl (1% of total cells) onto prewarmed LB+Kan and Iso+Kan+Tmp plates

For "-ligase" control reactions:

21A) Make a 1:1000 dilution of recovered cells (3μl of the 1:10 diluted cells + 297μl Isosensitest). Plate 90μl (0.01% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.

For "+ligase" reactions:

21B) Make a 1:10,000 dilution of recovered cells (1μl of the 1:10 diluted cells + 999μl Isosensitest). Plate 90μl (0.001% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.

22) Transfer remainder of the "+ligase" reaction to a flask containing 150ml Isosensitest. Add 300μl 500x Tmp stock.
23) Grow plates and culture at 37°C overnight.
24) The following morning, pull plates and count colonies. Calculate the number of independent transformants in your "+ ligase" pools.
25) Make a glycerol frozen stock of the "+ ligase" overnight culture. Store at -80°C.
26) Pellet the remainder of the "+ ligase" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 37 directly.
27) Purify the DNA from the "+ ligase" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool after removal of Tn7L ("xpT-L").

Remove Tn7R end with *Pme*I digest

21) Digest 1 μg of xpT-L pool with *Pme*I; use 100ng of xpT-L pool for a "no enzyme" control.

No enzyme	***Pme*I**	
100 ng	1000 ng	xpT pool DNA
1 μl	1 μl	10x NEB buffer #4
1 μl	1 μl	10x BSA
0 μl	0.5 μl	*Pme*I
To 10μl total	To 10μl total	dH2O

22) Incubate restriction digests and control reactions at 37°C for 1hr.
23) Heat inactivate *Pme*I by incubating reactions at 65°C for 20 minutes.
24) Run all of the "no enzyme" control and 1μl (100ng of DNA) from *Pme*I digest on an agarose gel to confirm restriction digest was successful. The uncut control should contain supercoiled plasmid. The *Pme*I digest should have a high MW band of plasmid backbone DNA, and the released Tn7R end should be visible at 863bp. If successful, continue with protocol.

Clean digest prior to ligation

We found that residual PmeI may interfere with the success of the ligation reaction.

25) Add ddH2O to remaining *Pme*I-digested xpT-L material to bring final volume to 100μl.
26) Add 100μl phenol:chloroform:IAA. Vortex to mix.
27) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Remove and discard organic (bottom) layer.
28) Add 100 μl chloroform. Vortex to mix.
29) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Transfer aqueous (top) later to a new 1.5ml microfuge tube.
30) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
31) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
32) Wash DNA pellet with 500μl ice-cold 70% EtOH.
33) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
34) Resuspend DNA pellet (may not be visible) in 20μl of 10mM Tris pH 7.5.

Ligate to recircularize plasmid after *Pme*I digestion

Longer overnight reaction is performed to encourage ligation of blunt ends from PmeI digest.

35) Set up ligation reaction (and a negative control) as shown:

- control	**+ ligase**	
10 μl	10 μl	DNA (200ng)
5 μl	5 μl	10x T4 ligase buffer
5 μl	5 μl	10mM ATP
0 μl	2.5 μl	T4 DNA ligase
30 μl	27.5 μl	dH2O
50 μl	50 μl	TOTAL

36) Ligate at 16°C, 16 hours.
37) Heat inactivate ligase enzyme by incubating at 65°C, 20 minutes

Clean the ligated DNA after removal of Tn7R

38) Add 50μl ddH2O to the ligated DNA, to bring it to a final volume of 100μl.
39) Add 10 μl 3M NaOAc. Add 220μl ice-cold 100% EtOH. Chill at -20°C for 15 minutes.
40) Spin 15 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant.
41) Wash DNA pellet with 500μl ice-cold 70% EtOH.
42) Spin 5 minutes, 4°C, 13500 rpm in microfuge. Discard supernatant. Allow pellet to air dry.
43) Resuspend DNA in 4μl 10mM Tris. DNA will be roughly 50ng/μl

Transform ligated DNA after removal of Tn7R

For both "- control" and "+ ligase" reactions:

44) Combine 1μl of DNA (~ 112ng/μl) with 20μl MegaX cells in a chilled electroporation cuvette.
45) Electroporate at 2.0 kV, 200Ω, 25μF, according to protocol.
46) Add 1ml Isosensitest to the cuvette and pipet up and down to resuspend.
47) Transfer 900μl cells to a 1.5ml microfuge tube.

For "-ligase" control reactions:

48A) Recover at 37°C for 1 hour in microfuge tube.
49A) Make 1:10 dilution of recovered cells (30μl cells + 270μl LB). Plate 90μl (1% of total cells) onto prewarmed LB+Kan and Iso+Tmp+Kan plates.
50A) Make a 1:100 dilution of recovered cells (40μl of 1:10 dilution of cells, 360μl LB). Plate 90μl (0.1% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates
51A) Make a 1:1000 dilution of recovered cells (40μl of 1:100 dilution of cells, 360μl LB). Plate 90μl (0.01% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.
52A) Grow plates at 37°C overnight

For "+ligase" control reactions:

48B) Transfer the cells to a 500ml flask containing 150mls LB. Recover at 37°C for 1 hour.
49B) Plate 150μl of recovered cells (0.1% of total cells) onto prewarmed LB+Kan and Iso+Tmp+Kan plates.
50B) Make a 1:10 dilution of recovered cells (60μl of cells, 540μl LB). Plate 150μl (0.01% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates
51B) Make a 1:100 dilution of recovered cells (50μl of 1:10 dilution of cells, 450μl LB). Plate 150μl (0.001% of total cells) onto prewarmed LB+Car, Iso+Tmp, and Iso+Tmp+Car plates.
52B) Add 150μl 100x Car stock to culture flask. Grow plates and flask at 37°C overnight

53) The following morning, pull plates and count colonies. Calculate the number of independent transformants in your "+ ligase" pools.
54) Make a glycerol frozen stock of the "+ ligase" overnight culture. Store at -80°C.
55) Pellet the remainder of the "+ ligase" overnight culture in a 250ml conical tube. Spin 15 minutes at 3000 rpm. Pellet can be stored at -20°C, or continue to step 37 directly.
56) Purify the DNA from the "+ ligase" culture cells using a Qiagen HiSpeed Midiprep Kit. Elute in 1ml 10mM Tris. This is the expression pool after removal of both Tn7L and Tn7R("xpT-L-R").

Diagnostic digests

We highly recommend performing diagnostic restriction digests on plasmid pools from each phase of this protocol. This helps to characterize the proportion of each pool that is mutagenized, identify any lingering donor plasmid that persists in the expression pools, and helps determine the complexity of the pool (ie, is the Tn inserted at many locations throughout the target ORF?).

We suggest performing many restriction digests in parallel. Use an enzyme that cuts only in the donor backbone, one that cuts only in the expression backbone, one that cuts in the Tn7L, and one that cuts in Tn7R. Perform "no enzyme" controls, as well. Include one double digestion which cuts in the epitope tag and one in the backbone – this will determine the complexity of the mutagenized pool. Perform restriction digest on the following DNA samples:

1) Unmutagenized target donor vector
2) wt expression vector
3) mutagenized donor pool
4) xpC pool
5) xpT pool
6) xpT-L pool
7) xpT-L-R pool

References

1. Brachmann, C.B. et al. Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast* **14**, 115-132 (1998).
2. Winzeler, E.A. et al. Functional characterization of the S. cerevisiae genome by gene deletion and parallel analysis. *Science* **285**, 901-906 (1999).
3. Gamas, P. & Craig, N.L. Purification and characterization of TnsC, a Tn7 transposition protein that binds ATP and DNA. *Nucleic acids research* **20**, 2525-2532 (1992).
4. Choi, K.Y., Li, Y., Sarnovsky, R. & Craig, N.L. Direct interaction between the TnsA and TnsB subunits controls the heteromeric Tn7 transposase. *Proceedings of the National Academy of Sciences of the United States of America* **110**, E2038-2045 (2013).

File S2

Calculating fold coverage of Tn-mutagenized pools.

For entry pools: Fold coverage was calculated by dividing the number of Tmp^RKan^R transformants by the length of plasmid DNA "available" for mutagenesis. The target plasmid, *DCW1* entry vector, is 3608bp long. When considering the portions of the entry vector into which we could expect insertions, we excluded the origin (683 bp) and Kan^R gene (807bp), because plasmids with insertions in these regions would not be expected to propagate. Thus, 4236bp (2118 bp x 2 strands of DNA) of sequence in *DCW1* entry vector was available for mutagenesis. We observed 2.01×10^5 Tmp^RKan^R colonies / 4236bp = 47-fold coverage.

For expression pools: There are 1373 bp between Gateway recombination sites in a wild type *DCW1* expression vector. In our mutagenized expression pools, all Tn7 insertions will be present in this area, since that is the only region mobilized from the mutagenized *DCW1* entry pool by Gateway recombination. Because the Tn7 can insert in either direction, we consider the "available" DNA for mutagenesis in these constructs to be 2746 bp long. Fold coverage is calculated using the number of Tmp^RCar^R colonies in the expression pools, or Car^R colonies in the final pool. See Supplementary Note 3 for a discussion of how the number of Tmp^RCar^R colonies was calculated in the xpC pool. We observed 91x fold coverage in the initial expression pool (xpC), and recovered sufficiently numbers of transformants in subsequent steps to maintain this coverage. As the initial mutagenesis of the *DCW1* entry vector had 47x coverage, these expression pools are sufficiently large to maintain the complexity present in the initial mutagenized entry pool.

File S3

Revising xpC pool sizes

To properly assess the size of the xpC pool, we found it was necessary to plate on selective media both pre-and post- Car selection. We suspect the MegaX *E. coli* cells are transformed with multiple plasmids and do not segregate them properly; thus the need to distinguish between cells that carry Tmp^R and Car^R on a single plasmid (as desired), and those that carry it on separate plasmids. Because of the possibility the strain carries multiple plasmids, co-selection with Tmp and Car may overestimate the true number of mutagenized expression plasmids in the pool (as counted from Iso+Tmp+Car plates). Theoretically, by performing the Car and Tmp selection sequentially, we minimize the selective pressure for MegaX cells to maintain multiple plasmids.

First, to assess the transformation efficiency, recovered cells were plated (prior to Car selection overnight) and found to contain $5x10^6$ Car^R colonies. This represents the number of independent expression vectors (mutagenized or not) in the xpC pool. Second, to determine how many of these Car^R colonies are also Tmp^R, we took a portion of the cells from the xpC pool (post-Car selection) onto LB+Car plates, and then replica plated to various selective media to determine the precise drug-resistance of each colony (Table S5).

In detail: cells from the xpC frozen stock were resuspended in PBS, diluted, and plated to get roughly 200 colonies per LB+Car plate; 4 LB+Car plates were used. These plates were grown overnight at 37°C. Each LB+Car plate was then replica plated (in order) to LB+Kan, Iso+Tmp+Kan, Iso+Tmp, Iso+Tmp+Car, and LB+Car plates, and grown at 37°C overnight. The total number of colonies were counted on each plate. Additionally the Iso+Tmp+Car and Iso+Tmp+Kan were compared to identify any colonies resistant to Tmp, Car, and Kan. This would suggest a single cell contains two plasmid populations, indicating sub-optimal plasmid segregation in MegaX cells.

Calculations for drug resistance of colonies were performed as follows:

$Tmp^RKan^RCar^R$ colonies = # colonies on Iso+Tmp+Kan plate (is Car^R, based on growth on source plate).

Tmp^RKan^R colonies = (colonies on Iso+Tmp+Kan plates) – (# of $Tmp^RKan^RCar^R$ colonies)

Tmp^RCar^R colonies = (colonies on Iso+Tmp+Car plates) –(# of $Tmp^RKan^RCar^R$ colonies)

Kan^RCar^R colonies = (# colonies on LB+Kan plate) – (# of $Tmp^RKan^RCar^R$ colonies) – (# of Tmp^RKan^R colonies)

Car^R only colonies = (# colonies on LB+Car plate) – (# of $Tmp^RKan^RCar^R$ colonies) – (# of Tmp^RCar^R colonies)-(# of Kan^RCar^R colonies).

See results of calculations in Table S5. The correction factor was calculated as the Tmp^RCar^R/ (total Car^R), as we selected for only Car^R in the culture and we can assume we counted the LB+Car plates accurately. "total Car^R" would be the sum of ($Tmp^RKan^RCar^R$), (Tmp^RCar^R), (Kan^RCar^R) and (Car^R only) colonies. We found that an average 5% of the Car^R colonies in the xpC culture were actually Tmp^RCar^R. Thus, 5% of the $5x10^6$ Car^R transformants counted would estimate our Tmp^RCar^R population to be $2.5x10^5$ Tmp^RCar^R colonies in the xpC pool, as shown in Table 1.

File S4

Assessing pool complexity

Restriction digests were used diagnostically at each step of the mutagenesis and processing of the mutagenized pool. This enabled us to monitor if donor vectors or unmutagenized expression plasmids were errantly maintained in the pool. Also, double digests with enzymes that cut in the backbone and in the epitope tag allowed us to qualitatively assess pool complexity. Digesting a mutagenized plasmid pool in this way should result in smeared bands after gel electrophoresis, where the range of fragment sizes should reflect epitope tags inserted throughout the target ORF (data not shown).