The Novo Nordisk Foundation
Center for Biosustainability

EASY CLONE 2.0

Integrative vector set for *Saccharomyces cerevisiae*

USER MANUAL

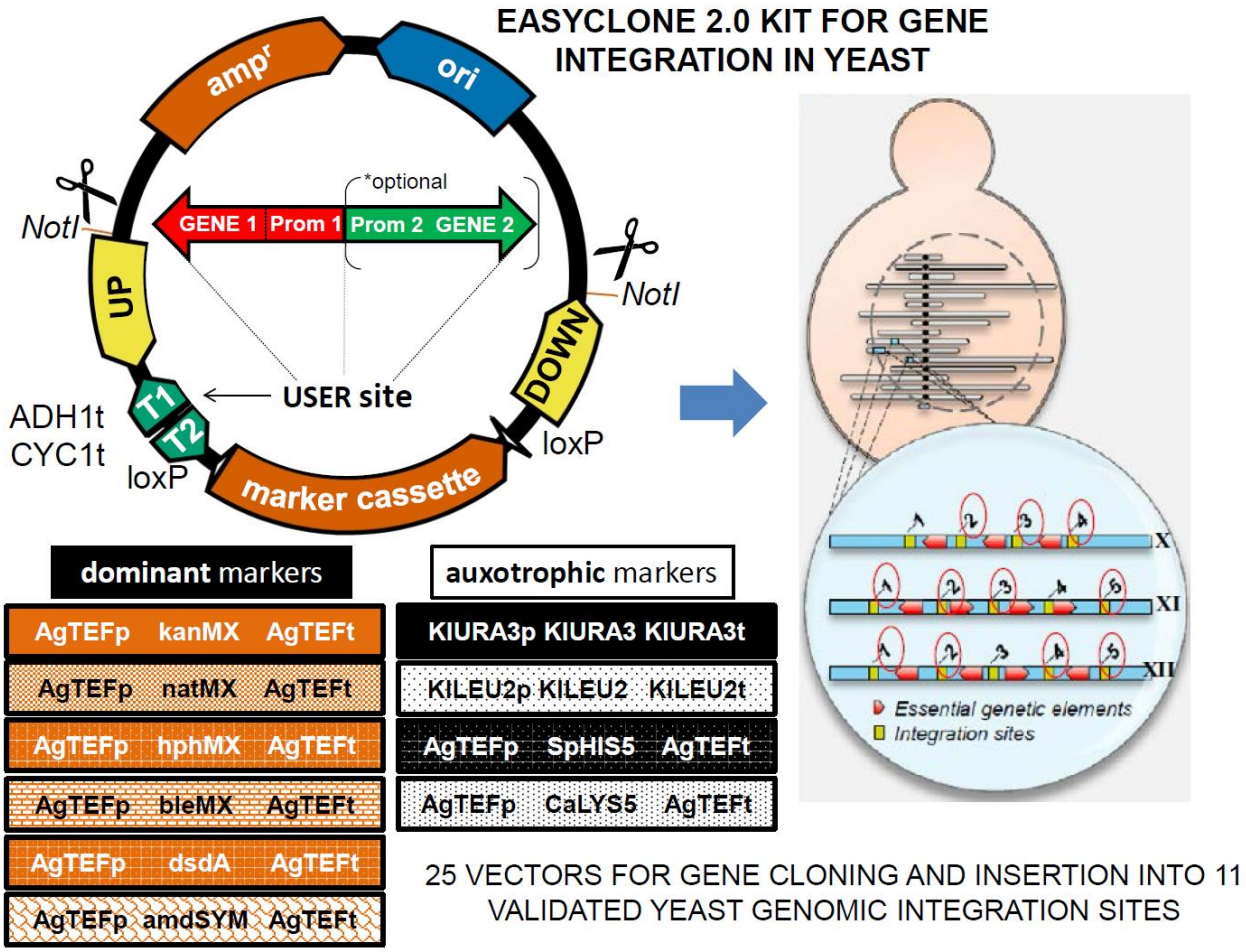
Table of Contents

INTRODUCTION

Overview	The collection contains a set of 25 integrative vectors suitable for (over-)expression of (heterologous) genes in both laboratory and industrial *S. cerevisiae* strains. The vectors allow for selection in auxotrophic yeast strains (four different auxotrophic markers) as well as in prototrophic yeast strains (six different dominant selection markers). The EasyClone system provides a possibility of cloning of up to two genes, when a bidirectional promoter (any of choice) is used and integration of the construct into well-defined 11 yeast chromosomal locations. Particular biobricks are assembled and cloned into the vectors via Uracil-Specific Excision Reaction (USER) cloning. The marker genes are flanked with loxP sites allowing for marker rescue and recycling, providing a possibility of repeated rounds of gene insertions.
References	EasyClone 2.0 is described in (Stovicek *et al.*, 2015a). EasyClone with detailed description of the cloning procedure (Jensen *et al.*, 2014). Original paper on characterization of integration sites (Mikkelsen *et al.*, 2012). USER cloning method https://www.neb.com/applications/cloning-and-synthetic-biology/user-cloning.
Related materials	tbd

LIST OF EASYCLONE 2.0 VECTORS

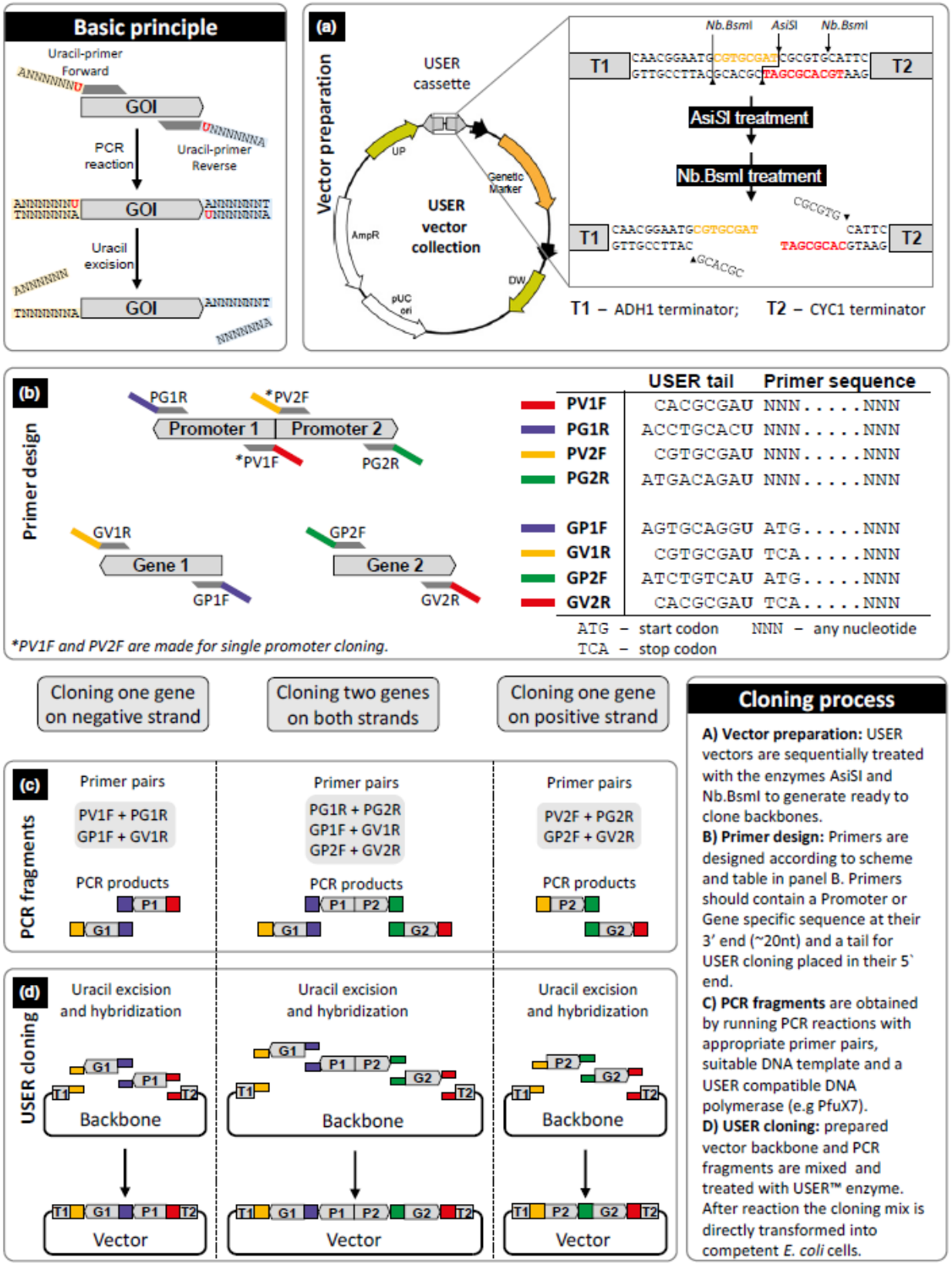
Vector characteristics	The EasyClone 2.0 vector set contains 25 integrative vectors with auxotrophic or dominant selection markers. The vectors are designed for integration into 11 specific loci on chromosomes X, XI and XII. The integration sites were chosen for high expression level of the inserted gene and for no interference with the cellular growth (Mikkelsen et al., 2012). The vectors contain USER site, flanked by ADH1 and CYC1 terminators, for cloning of genes and promoters. The cloning can be conveniently accomplished by USER cloning, however other methods can be used as well, such as in-fusion, Gibson, MoClo, etc. The vectors have *E. coli* origin of replication and ampicillin resistance for propagation in *E. coli*.
Storage	Upon receipt of the EasyClone 2.0 vector set, the plasmids must be transformed into *E. coli* and the transformants selected on LB-amp agar plates. Single colonies should be inoculated in liquid LB-amp overnight and the plasmids extracted from the cells, following the protocol outlined by plasmid extraction kit of choice.



Integration site	KlURA3	KlLEU2	SpHIS5	CaLYS5	natMX	kanMX	hphMX	ble	amdSYM	dsdAMX
X-2	pCfB2188				pCfB2193					
X-3		pCfB2189				pCfB2223				
X-4			pCfB2226				pCfB2194			
XI-1	pCfB2374				pCfB2375					
XI-2		pCfB2190				pCfB2224				
XI-3			pCfB2227				pCfB2195			
XI-5				pCfB2229				pCfB2196	pCfB2399	
XII-1	pCfB2191				pCfB2197					pCfB2400
XII-2		pCfB2192				pCfB2225				
XII-4			pCfB2228				pCfB2198			
XII-5			pCfB2336				pCfB2337			

METHOD OVERVIEW

Selection of vectors	For simultaneous transformation of several vectors, the vectors must have different selection markers. Once a particular integration site is occupied, the strain cannot be transformed with another expression vector targeting the same site, because it will replace the originally integrated expression vector. To obtain maximum targeting efficiency the vectors for simultaneous transformations should preferentially be chosen in such a way that they integrate into different chromosomes. In the opposite case some of the yeast transformants may have some expression vectors integrated in a wrong location. When using diploid strains, it can be expected that the expression vectors will integrate on both allelic chromosomes, resulting in two copies per genome.
Cloning	The vectors are well suited for cloning of one or two genes into the USER cloning site. The genes can be either native or heterologous. The promoters can be either native or synthetic. Native promoters and genes can be amplified from yeast genomic DNA. Vectors with bi-directional double promoter cassettes can be obtained from (tbd). The cloning process consists of: a) vector preparation, b) primer design, c) generation of gene and promoter biobricks via PCR, d) USER cloning. The vectors should be confirmed by sequencing. The procedures are described in details on the p. 7-11.
Transformation into yeast	Using vectors with auxotrophic selection markers, one to three vectors can be transformed into yeast simultaneously. Using vectors with dominant selection markers, one to two vectors can be transformed into yeast simultaneously. For some industrial *S. cerevisiae* strains, which show low transformation efficiencies, it may be possible only to transform one expression vector per transformation. The transformation procedure is described on p. 13.
Removal of the selection markers	If required, selection markers can be removed from the yeast strains using Cre-LoxP recombination (p. 14). For this, the strains need to be transformed with an episomal vector with CreA recombinase under control of GAL1 promoter (http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/Del_plas.html), the CreA expression is induced and the clones are plated and screened for the loss of the selection markers and the CreA vector.


Basic principle
Uracil-primer Forward
GOI
PCR reaction
Uracil-primer Reverse
Uracil excision
(a)
Vector preparation
USER cassette
USER vector collection
UP
Genetic Marker
DW
pUC ori
AmpR
Nb.BsmI
AsiSI
Nb.BsmI
T1
T2
AsiSI treatment
Nb.BsmI treatment
T1 – ADH1 terminator; T2 – CYC1 terminator
(b)
Primer design
PG1R
*PV2F
Promoter 1
Promoter 2
*PV1F
PG2R
GV1R
GP2F
Gene 1
Gene 2
GP1F
GV2R
USER tail
Primer sequence
PV1F CACGCGAU NNN.....NNN
PG1R ACCTGCACU NNN.....NNN
PV2F CGTGCGAU NNN.....NNN
PG2R ATGACAGAU NNN.....NNN
GP1F AGTGCAGGU ATG.....NNN
GV1R CGTGCGAU TCA.....NNN
GP2F ATCTGTCAU ATG.....NNN
GV2R CACGCGAU TCA.....NNN
ATG – start codon
TCA – stop codon
NNN – any nucleotide
*PV1F and PV2F are made for single promoter cloning.
Cloning one gene on negative strand
Cloning two genes on both strands
Cloning one gene on positive strand
(c)
PCR fragments
Primer pairs
PV1F + PG1R
GP1F + GV1R
PG1R + PG2R
GP1F + GV1R
GP2F + GV2R
PV2F + PG2R
GP2F + GV2R
PCR products
P1
P2
G1
G2
(d)
USER cloning
Uracil excision and hybridization
Backbone
Vector
Cloning process
A) Vector preparation: USER vectors are sequentially treated with the enzymes AsiSI and Nb.BsmI to generate ready to clone backbones.
B) Primer design: Primers are designed according to scheme and table in panel B. Primers should contain a Promoter or Gene specific sequence at their 3' end (~20nt) and a tail for USER cloning placed in their 5` end.
C) PCR fragments are obtained by running PCR reactions with appropriate primer pairs, suitable DNA template and a USER compatible DNA polymerase (e.g PfuX7).
D) USER cloning: prepared vector backbone and PCR fragments are mixed and treated with USER™ enzyme. After reaction the cloning mix is directly transformed into competent E. coli cells.

PROTOCOLS

Preparation of vectors for USER cloning

Digestion with AsiSI/SfaAI

The EasyClone 2.0 vectors are linearized with SfaAI (AsiSI), e.g. FastDigest® SfaAI from ThermoScientific (p. 6, a).

1. Prepare the reactions as following:
   X µl of EasyClone 2.0 vector (20 µg)
   20 µl of FastDigest® buffer
   5 µl of FastDigest SfaAI® restriction enzyme
   Water to a final volume of 200 µl
2. Incubate for 1 hour at 37°C.
3. Purify the plasmid from solution, using e.g., NucleoSpin® Gel and PCR Clean-up from Macherey Nagel, eluting with 50 µl of elution solution.
4. Determine DNA concentration using Nanodrop or sim.

Proceed to nicking step.

Nicking with Nb.BsmI

The vectors linearized with SfaAI (AsiSI) are nicked with Nb.BsmI, e.g. from New England Biolabs Inc.

1. Prepare the reactions as following:
   40 µl of SfaAI-digested vector
   X µl of Nb.BsmI (use 1U per 1 µg of vector)
   5 µl of buffer NEB 3.1
2. Incubate for 1 hour at 65°C (best use PCR machine and heated lid protocol, otherwise the water will evaporate from the reaction mix and condense on the lid).
5. Purify the digested and nicked vector from the gel, using e.g., NucleoSpin® Gel and PCR Clean-up from Macherey Nagel. Elute with 50 µl of elution solution.
3. Determine DNA concentration using Nanodrop or sim.
4. Store the USER-ready vectors at -20°C for repeated use.

Proceed to USER cloning.

Primer design

Primers with uracil overhangs

Design primers for amplification of your genes and promoters of interest with the appropriate overhangs (p. 6, b).
The primers must contain uracils and can be ordered from commercial oligo suppliers, e.g., IDT DNA.

The primers should contain the following overhangs:

Gene 1 primers
forward primer (GP1F): **AGTGCAGGU** *AAAACA* ATG$(N)_n$
reverse primer (GV1R): **CGTGCGAU** TCA$(N)_n$

Gene 2 primers
forward primer (GP2F): **ATCTGTCAU** *AAAACA* ATG$(N)_n$
reverse primer (GV2R): **CACGCGAU** TCA$(N)_n$

Promoter 1 primers
forward primer (PV1F): **CACGCGAU**$(N)_n$
reverse primer (PG1R): **ACCTGCACU(**$(N)_n$

Promoter 2 primers
forward primer (PV2F): **CGTGCGAU**$(N)_n$
reverse primer (PG2R): **ATGACAGAU**$(N)_n$

USER overhang in bold, *AAAACA* – Kozak sequence, ATG – start codon, TCA – stop codon, $(N)_n$ – gene(promoter)-specific sequence.

Preparation of DNA BioBricks

Uracil-tolerant polymerases

BioBricks, encoding genes and promoters, are amplified with primers containing uracils (p. 6, c). It is necessary to use a DNA polymerase that can read through uracils present in the primers and that has proofreading activity, e.g., Phusion U Hot Start DNA Polymerase from ThermoFisher Scientific or PfuTurbo Cx Hotstart DNA Polymerase from Agilent. Follow the manufacturer's instructions for the PCR conditions.

PCR-amplification of BioBricks

1. Prepare the PCR reactions as following:
   32 µl of water
   10 µl of 5X Phusion HF Buffer
   2.5 µl of forward primer (10 µM)
   2.5 µl of reverse primer (10 µM)
   1 µl of dNTP mix (10 mM)
   1 µl template DNA (plasmid or genomic DNA)
   1 µl of Phusion U Hot Start DNA Polymerase
2. Run the following PCR program:
   98°C for 1 min
   30 cycles of
   [98°C for 10 seconds
   54°C for 30 seconds (or another suitable annealing temperature)
   72°C for 1 min per 1 kb of the PCR product]
   72°C for 5 min
   10°C pause
3. Purify the BioBricks from the gel using e.g., NucleoSpin® Gel and PCR Clean-up from Macherey Nagel, eluting with 50 µl of elution solution. *Note: alternatively the BioBricks can be purified from solution or PCR reaction can be used directly. This can however result in lower cloning efficiency.*
4. Store the BioBricks at -20°C for repeated use.

Proceed to USER cloning.

USER cloning of BioBricks into vector

USER cloning and transformation into *E. coli*

The Biobricks are cloned into EasyClone 2.0 vectors, treated with SfaAI/Nb.BsmI (p. 6, d).

1. Prepare the USER reaction as following:
   1 µl of SfaAI/Nb.BsmI-treated vector*
   1 µl of BioBrick for gene 1 use vector to insert molar ratio 1:3
   1 µl of BioBrick for gene2
   1 µl of BioBrick for double promoter
   0.5 µl 5xHF buffer (NEB)
   0.5 µl USER™ enzyme (NEB)

   Note: when cloning only gene1 or gene2, use water instead of the missing BioBrick.

   *(*Optional step – you can test the quality of the vector digestion before you proceed to the cloning of a gene of interest*
   - Clone a gene encoding green fluorescent protein suitable for expression in E. coli into a SfaAI/Nb.BsmI-treated vector
   - The GFP gene can be amplified from p1976 (pUC19-GFP-cassette) vector with the following primers: CGTGCGAUCCGCATAGGGAGTGTAAATTTATC
   CACGCGAUAGTGAAAGGAAGGCCCATGAG
   - After you have finished the steps 2 and 3, evaluate the number of background (white) transformants on plates under blue light bench top illuminator – at least 80% of positive (green) colonies should be obtained)

2. Incubate the mixture in PCR machine at the following conditions:
   37°C for 25 min
   25°C for 10 min
   20°C for 10 min
   15°C for 10 min
   10°C pause
3. Transform the reaction mix into competent *E. coli* cells.
   - Cool the tubes on ice and add 95 µl of competent *E. coli* DH5α cells
   - After 10 min on ice, perform heat shock at 42°C for 90 s and place the tubes on ice for 1-2 min
   - Add 50 µl SOC media to each tube and incubate at 37°C incubator for 30 min
   - Plate the cells on LB-amp plates and incubate at 37°C overnight.

Proceed to verification of expression vector assembly.

Verification of expression vector assembly by colony PCR

Verification primers

The successful cloning of genes and promoters into the EasyClone 2.0 vectors can be identified by PCR on *E. coli* colonies.
If the total size of the cloned fragments does not exceed 5 kb, then the following primers can be used:

- ADH1_test_fw: GAAATTCGCTTATTTAGAAGTGTC
- CYC1_test_rv: CTCCTTCCTTTTCGGTTAGAG

In the opposite case a pair of primers can be used to verify cloning of gene1: forward primer used for amplification of the gene1 and ADH1_test_fw as a reverse primer
and cloning of gene2: forward primer used for amplification of the gene2 and CYC1_test_rv as a reverse primer

***E. coli* colony PCR**

1. Mix the following in a PCR tube (for 10 reactions of 10 µl each):
   50 µl 2xOneTaq Master Mix (New England Biolabs)
   10 µl µM forward verification primer
   10 µl µM reverse verification primer
   30 µl water
2. Add small amount of *E. coli* colony biomass (it is enough to touch the colony with a tip) to each PCR tube.
3. Run the following PCR program:
   94°C for 3 min
   35 cycles of
   [94°C for 20 seconds
   50°C for 30 seconds (or another suitable annealing temperature)
   68°C for 1 min per 1 kb of the PCR product]
   68°C for 5 min
   10°C pause
4. Analyze the PCR reactions on 1% agarose gel or on LabChip GXII (Caliper).

Plasmid purification and sequencing

1. Inoculate the *E. coli* clones containing correct vectors into 3-5 ml of liquid LB medium with ampicillin and cultivate overnight at 37°C.
2. Preserve the *E. coli* for future use: mix 500 µl of overnight *E. coli* culture with 500 µl of 50% v/v sterile glycerol solution. Store in cryotubes at -80°C.
3. Use the rest of the overnight culture to purify the vectors (e.g. NucleoSpin kit from Macherey-Nagel).
4. Sequence the cloned inserts.

Proceed to vector linearization and transformation.

Linearization of expression vector

Choice of restriction enzyme for linearization

Before transformation into yeast, the constructed expression vectors are linearized. Linearization of the vectors promotes integration into chromosome via homologous recombination. The vector fragments are integrated via double cross-over events of yeast chromosome with the UP and DOWN regions, flanking the expression cassette and the selection marker.

The DNA fragment targeted for integration can be excised from the expression vector by digestion with NotI.
If however any of the cloned genes or promoters contain NotI recognition sequence (GC^GGCC_GC), then other restriction enzymes should be used, e.g., SmiI (SwaI).

Linearization with NotI

1. Mix the following:
   X µl of expression vector (min 1 µg)
   5 µl of FastDigest® buffer
   X µl of FastDigest NotI® (use 0.2 µl per 1 µg DNA)
   water up to 50 µl
2. Incubate at 37°C for 1 hour.
3. (Optional step) Confirm linearization on the gel and if desired purify the correct fragment from the gel (be aware of the *E. coli* backbone fragment of 2.8 kb).
4. Purify the linearized vector from solution using e.g., NucleoSpin® Gel and PCR Clean-up from Macherey Nagel. Store at -20°C until transformation.

Transformation of expression vector(s) into yeast (according to Gietz and Woods, 2002)

Preparation of competent *S. cerevisiae* cells

1. Inoculate the yeast strain into 10 ml of YPD or SC selection medium and grow overnight at 30°C in shaker.
2. Determine the titer of the yeast culture and inoculate 2.5 x 10^8 cells into 50 ml of fresh YPD medium to give 5x10^6 cells per ml of the culture.
3. Grow the culture for approx. 4 hours to get the cell titer 2x10^7 cells per ml
4. Harvest the cells by centrifugation, wash in 25 ml of sterile water and resuspend in 1 ml water.
5. Transfer the cell suspension to a 1.5 ml microcentrifuge tube, spin down for 30 sec and discard the supernatant.
6. Add water to a final volume of 1.0 ml and vortex mix vigorously to resuspend the cells.
   Note: If the cell count of the culture is greater than 2 x 10^7 cells per ml the volume then increase the volume to maintain the titer of this suspension at 2 x 10^9 cells per ml. If the cell count of the culture is less than 2 x 10^7 cells/ml then decrease the volume.
7. Pipette 100 µl samples (10^8 cells) into 1.5 ml microfuge tubes, one for each transformation, centrifuge at top speed for 1 min and remove the supernatant.

Proceed to yeast transformation.

Yeast transformation and selection

1. Mix the following Transformation mix sufficient:
   240 µl 50% (w/v) Polyethylene glycol MW 3350 (PEG)
   36 µl 1M Lithium Acetate (LiAc)
   10 µl ss-carrier DNA (10 mg/ml) – boil for 3 min before use and keep on ice
   74 µl linearized vector (0.1- 1 µg) + water
2. Keep the Transformation Mix in ice water until mixed with competent cells.
3. Add 360 µl of Transformation Mix to each transformation tube and resuspend the cells by vortex mixing vigorously.
4. Incubate the tubes in a 42°C water bath for 40 min.
5. Spin down the cells, remove the supernatant and either plate the cells on selective medium (SC lacking one of the following compounds – histidine, uracil, leucine, lysin – see Media recipes p. 17) when auxotrophic selection is used, or resuspend the cells in 1 ml YPD when dominant selection is used
6. Incubate the cells in YPD for 2 hours at 30°C with shaking to provide time for expression of a resistance gene.
7. Spin down the cells, resuspend in water and plate on selective plates (see Media recipes p. 17).

Proceed to verification of correct vector integration in the yeast genome.

Removal of the selection markers by Cre-LoxP recombination (according to Hegemann and Heick, 2011)

CreA vectors

For selection of a suitable CreA-expressing vector see http://web.uni-frankfurt.de/fb15/mikro/euroscarf/data/Del_plas.html

Transformation with CreA vector and induction

1. Transform the cells with a CreA-expressing vector using the procedure described above (p. 13).
2. Plate the transformed cells (plate also 10x and 100x diluted cell suspension) on corresponding selection plates (selection for a marker on the CreA-carrying vector)
3. Incubate the plates for 2-3 days at 30°C
4. For induction of CreA-expression, inoculate single colonies into 10 ml of YPGalactose medium (supplemented with an appropriate antibiotic to keep the selection pressure, see Media recipes p. 17) overnight.
5. Determine the number of cells in the culture and plate 100-200 cells onto YPD plates and incubate them for 2 days at 30°C.

Proceed to testing for the removal of selection markers

Testing for the removal of selection markers and CreA vectors

1. Replica-plate onto plates selective for the marker(s) on the integrated EasyClone vector(s). Alternatively, about 12 colonies can be streaked onto the selective plate(s).
2. Cells that fail to grow on the selective medium have lost the marker cassette(s).
3. Pick the cells from the corresponding colonies on YPD plates and streak on fresh YPD plate.
4. To remove the Cre expression plasmid from a marker lacking yeast strain, incubate the cells in 10 mL of YPD medium (non-selective) overnight.
5. Plate 100-200 cells onto YPD plates and incubate for 2 days at 30°C.
6. Replica-plate onto plates selective for the marker carried on CreA-expressing vector. Alternatively, about 12 colonies can be streaked out onto the selective plate.
7. The cells that cannot grow on the selective medium have lost the CreA-expressing vector.
8. Pick the cells from the corresponding colonies on YPD plates and streak on fresh YPD plate.
9. Finally, test again for loss of the EasyClone marker gene cassette(s) and the CreA-expressing vector by streaking the cells onto the selective plates.

APPENDIX

Verification of correct vector integration in the genome by yeast colony PCR

Verification primers and expected fragment sizes

Select suitable primers from the table below for verification of a correct insertion of your construct.

Note: Primers 2220 and 2221 are universal primers annealing to any vector of choice. The other primers are from a particular genomic region. Either one or both primer pairs can be used for verification of the particular correct integration.

Site	Primer ID	Sequence of the primer 5´-3´	Description	Fragment size (bp)
X-2	2220	CCTGCAGGACTAGTGCTGAG	X-2 DOWN	973
	902	GAGAACGAGAGGACCCAACAT		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	X-2 UP	873
	901	TGCGACAGAAGAAAGGGAAG		
X-3	2220	CCTGCAGGACTAGTGCTGAG	X-3 DOWN	667
	904	CCGTGCAATACCAAAATCG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	X-3 UP	1059
	903	TGACGAATCGTTAGGCACAG		
X-4	2220	CCTGCAGGACTAGTGCTGAG	X-4 DOWN	656
	906	GACGGTACGTTGACCAGAG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	X-4 UP	983
	905	CTCACAAAGGGACGAATCCT		
XI-1	2220	CCTGCAGGACTAGTGCTGAG	XI-1 DOWN	784
	908	GAAGACCCATGGTTCCAAGGA		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XI-1 UP	791
	907	CTTAATGGGTAGTGCTTGACACG		
XI-2	2220	CCTGCAGGACTAGTGCTGAG	XI-2 DOWN	818
	910	GAGACAAGATGGGGCAAGAC		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XI-2 UP	963
	909	GTTTGTAGTTGGCGGTGGAG		
XI-3	2220	CCTGCAGGACTAGTGCTGAG	XI-3 DOWN	704
	912	CACATTGAGCGAATGAAACG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XI-3 UP	927
	911	GTGCTTGATTTGCGTCATTC		
XI-5	2220	CCTGCAGGACTAGTGCTGAG	XI-5 DOWN	701
	8419	GCATGGTCACCGCTATCAGC		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XI-5 UP	1050
	8418	CTCAATGATCAAAATCCTGAATGCA		
XII-1	2220	CCTGCAGGACTAGTGCTGAG	XII-1 DOWN	896
	892	GGACGACAACTACGGAGGAT		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XII-1 UP	852

	891	CTGGCAAGAGAACCACCAAT		
XII-2	2220	CCTGCAGGACTAGTGCTGAG	XII-2 DOWN	666
	894	GGCCCTGATAAGGTTGTTG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XII-2 UP	795
	893	CGAAGAAGGCCTGCAATTC		
XII-3	2220	CCTGCAGGACTAGTGCTGAG	XII-3 DOWN	744
	896	TGGCCAATTGTTCAGTCAAG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XII-3 UP	963
	895	TGGGCAGCCTTGAGTAAATC		
XII-4	2220	CCTGCAGGACTAGTGCTGAG	XII-4 DOWN	667
	898	CGTGAAATCTCTTTGCGGTAG		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XII-4 UP	828
	897	GAACTGACGTCGAAGGCTCT		
XII-5	2220	CCTGCAGGACTAGTGCTGAG	XII-5 DOWN	799
	900	GTGGGAGTAAGGGATCCTGT		
	2221	GTTGACACTTCTAAATAAGCGAATTTC	XII-5 UP	811
	899	CCACCGAAGTTGATTTGCTT		

Yeast colony PCR

1. Check the transformation plates after 2-5 days of growth at 30°C.
2. Pick several clones and streak on selective plate.
3. Take small amount of fresh biomass and resuspend in 15 µl of 20 mM NaOH.
4. Incubate for 15 min at 96°C.
5. Vortex briefly and spin down the cells.
6. Mix the following in a PCR tube (for 10 reactions):
   50 µl 2xOneTaq Master Mix (New England Biolabs)
   10 µl µM primer 1
   10 µl µM primer 2
   20 µl water
7. Add 1 µl of the denatured biomass to each tube containing 9 µl of the PCR premix.
8. Run the following PCR program:
   94°C for 1 min
   35 cycles of
   [94°C for 20 seconds
   50°C for 30 seconds
   68°C for 1 min/kb of the PCR product]
   68°C for 7 min
   10°C pause
9. Analyze the samples on 1% agarose gel or on LabChip GXII (Caliper) (for corresponding PCR product size see the Table above).

Media recipes

YPD (+antibiotics)

Yeast Extract Peptone Dextrose Medium (1 liter)
1% yeast extract
2% peptone
2% dextrose (glucose or galactose)
± antibiotics for selection in the following concentrations:
200 mg/l G418 (kanMX selection marker)
200 mg/l Hygromycin B (hphMX selection marker)
100 mg/l Nourseothricin (natMX selection marker)
10 mg/l Phleomycin (bleMX selection marker)
± 2% agar

1. Dissolve the following in 900 ml of water:
   10 g yeast extract
   20 g of peptone
   (for plates) add 20 g of agar
2. Autoclave for 20 min at 120°C.
3. Cool solution to ~60°C and add 100 ml of 20% glucose.
4. For YPD agar, add antibiotics, if desired, and pour the plates.

Liquid YPD can be stored at room temperature. Antibiotics are added immediately prior to use.
YPD plates are stored at 4°C in the dark for up to one month.

SC

Synthetic Complete Medium
Yeast Nitrogen Base without aminoacids (Sigma-Aldrich catalogue number Y0626)
Yeast Synthetic Drop-out Medium Supplements
w/o uracil (Y1501)
w/o histidine (Y1751)
w/o lysine (Y1896)
w/o leucine (Y1376)
2% dextrose (glucose)
± 2% agar

For exact composition and protocol please visit the products' website

Liquid SC medium can be stored at room temperature. Plates should be stored at 4°C.

YNB-amd or D-ser

Yeast Nitrogen Base with acetamide or D-serine (1 liter)
0.17% yeast nitrogen base without aminoacids and ammonium sulfate
0.66% potassium sulfate
2% dextrose (glucose)
2% agar
0.06% acetamide (amdSYM selection marker)
or 0.2% D-serine (dsdA selection marker)

1. Dissolve the following in 800 ml of water:

1.7 g yeast nitrogen base without amino acids and ammonium sulfate
6.6 g potassium sulfate
20 g agar

7 Autoclave for 20 min at 120°C.
8 Cool the solution to ~60°C, add 100 ml of 20% glucose and 100 ml 0.6% acetamide or 100 ml 2% D-serine.
9 Poor the plates.

The plates should be stored at 4°C.

LB-amp

Lysogeny Broth Medium (1 liter)
1% tryptone
0.5% yeast extract
1% NaCl
1.5% agar for LB agar

1. Dissolve the following in 1 liter of water:
   10 g tryptone
   5 g yeast extract
   10 g NaCl
   for LB agar add 15 g agar
2. Autoclave for 20 min at 120°C.
3. Cool solution to ~60°C and add 100 mg/l ampicillin.
4. Poor the plates in case of LB agar medium.

The liquid as well as solid medium containing ampicillin should be stored at 4°C.

SOC

Super Optimal Broth (250 ml)
0.5 % yeast extract
2 % tryptone
10 mM NaCl
2.5 mM KCl
10 mM $MgCl_2$
10 mM $MgSO_4$
20 mM glucose

To make 250 mL of media:

1. Dissolve the following in 231 ml of water:
   1.25 g yeast extract
   5 g tryptone
   0.15 g NaCl
   0.005 g KCl
   0.51 g $MgCl_2$
2. Adjust pH to 7 by adding sodium hydroxide.
3. Autoclave the solution at 120°C for 20 min.
4. Let the solution cool and then add 10 ml of 20% glucose and 2.5 ml of 1M $MgSO_4$

Medium can be stored at room temperature.

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