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Yeast Functional Analysis Report

A suite of Gateway® cloning vectors for high-throughput genetic analysis in *Saccharomyces cerevisiae*

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Abstract

In the post-genomic era, academic and biotechnological research is increasingly shifting its attention from single proteins to the analysis of complex protein networks. This change in experimental design requires the use of simple and experimentally tractable organisms, such as the unicellular eukaryote *Saccharomyces cerevisiae*, and a range of new high-throughput techniques. The Gateway® system has emerged as a powerful high-throughput cloning method that allows for the *in vitro* recombination of DNA with high speed, accuracy and reliability. Two Gateway-based libraries of overexpression plasmids containing the entire complement of yeast open reading frames (ORFs) have recently been completed. In order to make use of these powerful resources, we adapted the widely used pRS series of yeast shuttle vectors for use in Gateway-based cloning. The resulting suite of 288 yeast Gateway vectors is based upon the two commonly used GPD and GAL1 promoter expression systems that enable expression of ORFs, either constitutively or under galactose-inducible conditions. In addition, proteins of interest can be fused to a choice of frequently used N- or C-terminal tags, such as EGFP, ECFP, EYFP, Cerulean, monomeric DsRed, HA or TAP. We have made this yeast Gateway® vector kit available to the research community via the non-profit Addgene Plasmid Repository (http://www.addgene.org/yeast_gateway).

Keywords: Gateway®; vector; high-throughput; cloning

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Introduction

Baker's yeast (*Saccharomyces cerevisiae*) is a powerful experimental system for studying complex biological processes. Notably, many of the key cellular pathways of yeast share a high degree of similarity to those of mammalian cells. In addition, the yeast genome is very well characterized and amenable to genetic manipulation. Methods are available to rapidly overexpress or knock out almost every gene, and efforts to generate a collection of haploid strains, each harbouring a single mutation, as well as a collection of expression plasmids with each yeast ORF under the control of an inducible promoter, have recently come to fruition (Cooper *et al*., 2006; Sopko *et al*., 2006). The availability of these tools turns yeast into a robust new system for investigating, on a genome-wide scale, the mechanisms underlying many cellular processes, with direct relevance to human disease and for the discovery of novel drug targets for therapeutic intervention.

A frustrating aspect of high-throughput screening is that it is often desirable to test candidate genes in multiple formats and/or combinations, but current cloning strategies usually make transferring ORFs from one vector backbone to another very time-consuming. To address this problem, we have constructed a suite of 288 yeast expression vectors compatible with the Gateway® recombination-based cloning system and describe here methods for their use in multiple applications. Our collection provides a choice of two promoters (constitutive or inducible), integrating or extra-chromosomal, high- or low-copy origins of replication, and the option for N- or C-terminal fusion to various protein affinity tags (HA, TAP) or fluorescent proteins (EGFP, ECFP, EYFP, Cerulean, DsRed). We have made these plasmids available to the research community (**http://www.addgene.org/yeast_gateway**) and we hope their use, along with the methods described here, will expedite the process of high-throughput screening and target validation.

Materials and methods

Vector construction

We first modified a set of 24 pRS yeast shuttle vectors (pRS303, pRS304, pRS305, pRS306, pRS413, pRS414, pRS415, pRS416, pRS423, pRS424, pRS425 and pRS426, each with a CYC1 terminator and a GPD or GAL1 promoter) to make them compatible for use with the Gateway® system. To generate this core set of pAG (Advanced Gateway) vectors, we inserted the chloramphenicol/ccdB resistance Gateway cassette A into the single *Sma*I restriction site of each pRS plasmid. Proper orientation of the Gateway cassette was confirmed by DNA sequencing. The resulting plasmids were then used to generate a set of derived vectors that allows for the expression of proteins with C-terminal tags (please see Supplementary Table 1 for nomenclature and a complete list of pAG plasmids). The coding sequences for the various tags were amplified using the primers and templates listed in Tables 1 and 2. PCR products were cloned between the *Hin*dIII and *Xho*I restriction sites of pAG426GPD and pAG426GAL (EGFP, ECFP, EYFP, Cerulean, DsRed and 3HA) or between the *Cla*I and *Xho*I sites of pAG423GPD and pAG423GAL (TAP). The correct amplification and integration of tag DNA sequences was confirmed by sequencing. To complete the set of pAG plasmids, the entire expression cassette was removed from the primary pAG423GPD/GAL-ccdB-tag and pAG426GPD/GAL-ccdB-tag constructs by digestion with *Sac*I and *Kpn*I and subcloned into the remaining pRS vector backgrounds, using *Sac*I and *Kpn*I for linearization of the target plasmids.

For the cloning of vectors for expression of N-terminal fusions, PCR-generated fragments (see Tables 1 and 2 for details) were inserted into the *Xma*I and *Xho*I sites of pRS426GPD and pRS426GAL. The resulting plasmids were cut with *Xho*I and incubated with mung bean nuclease to remove single-stranded overhangs. Next, the C1 Gateway cassette was ligated to the blunted plasmids. The resulting plasmids were subsequently analysed by sequencing to confirm the correct integration of tag sequences and Gateway cassette. As a final step, a *Spe*I–*Mlu*I fragment comprising the Gateway cassette and the tag sequence was cut out and cloned between the *Spe*I and *Mlu*I sites of the core set of pAG plasmids.

Table 1. Primers used for the amplification of tags

Primer	Sequence[a]
cEXFP-forw–*Hin*dIII	GCA GTA CGA AGC TTA ATG GTG AGC AAG GGC GAG GAG
cEXFP-rev–*Xho*I	GA TAG TGT CTC GAG TTA CTT GTA CAG CTC GTC CAT GCC G
DsRed-forw–*Hin*dIII	GCA GTA CGA AGC TTA ATG GAC AAC ACC GAG GAC GTC ATC AAG
DsRed-rev–*Xho*I	GA TAG TGT CTC GAG CTA CTG GGA GCC GGA GTG GCG G
TAP-forw–*Cla*I	GCA GTA CGA TCG ATA GGT GGA CCA GGT GGT GGA ATG AAG CGA CGA TGG AAA AAG
TAP-rev–*Xho*I	GA TAG TGT CTC GAG TCA CTG TTC TTT GCT CAC CGA AG
3HA-forw–*Hin*dIII	GCA GTA CGA AGC TTA GGT GGA ATG TAC CCA TAC GAT GTT CCT GAC T
3HA-rev–*Xho*I	GA TAG TGT CTC GAG TTA GCA CTG AGC AGC GTA ATC TG
nEXFP-forw–*Xma*I	G CAG CGT TCC CGG GAC AAA ATG GTG AGC AAG GGC GAG G
nEXFP-rev–*Xho*I	GA TAG TGT CTC GAG TCC ACC ACC TGG TCC ACC CTT GTA CAG CTC GTC CAT GCC G

[a] Restriction sites are underlined.

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Table 2. Primer combinations and templates used for the amplification of tags

Tag	Primer combination	Template
EGFP (C-terminal)	cEXFP-forw–*Hin*dIII cEXFP-rev–*Xho*I	pEGFP-1 (Clontech)
ECFP (C-terminal)	cEXFP-forw–*Hin*dIII cEXFP-rev–*Xho*I	pECFP-1 (Clontech)
EYFP (C-terminal)	cEXFP-forw–*Hin*dIII cEXFP-rev–*Xho*I	pEYFP-1 (Clontech)
Cerulean (C-terminal)	cEXFP-forw–*Hin*dIII cEXFP-rev–*Xho*I	pmCerulean-N1 (Rizzo *et al*., 2004)
DsRed M1 (C-terminal)	DsRed-forw–*Hin*dIII DsRed-rev–*Xho*I	pDsRed M1 (Clontech)
TAP (C-terminal)	TAP-forw–*Cla*I TAP-rev–*Xho*I	pVV220 (Van Mullem *et al*., 2003)
3HA (C-terminal)	3HA-forw–*Hin*dIII 3HA-rev–*Xho*I	pVV205 (Van Mullem *et al*., 2003)
EGFP (N-terminal)	nEXFP-forw–*Xma*I nEXFP-rev–*Xho*I	pEGFP-1 (Clontech)
ECFP (N-terminal)	nEXFP-forw–*Xma*I nEXFP-rev–*Xho*I	pECFP-1 (Clontech)
EYFP (N-terminal)	nEXFP-forw–*Xma*I nEXFP-rev–*Xho*I	pEYFP-1 (Clontech)
Cerulean (N-terminal)	nEXFP-forw–*Xma*I nEXFP-rev–*Xho*I	pmCerulean-N1 (Rizzo *et al*., 2004)

Standard BP and LR reaction protocol

We have developed modified versions of the standard Gateway LR and BP reactions. The smaller volumes require less enzyme, reducing the cost per reaction.

BP or LR reaction

- 150 ng expression clone (BP) or entry clone (LR): 1 l.
- 150 ng entry vector (BP) or destination vector (LR): 1 l.
- 1X TE (pH 8): 2 l.
- BP (or LR) Clonase II mix (Invitrogen): 1 l.

Incubate at room temperature for 1 h. Transform 2–3 l of the reaction into DH5α, TOP10 or other ccdB-sensitive cells. Select for transformants on LB agar plates containing 100 g/ml ampicillin.

Experiments with α-synuclein

Entry clones containing full-length human α-synuclein with or without a stop codon were used in Gateway LR reactions to generate 426GAL-α-syn-EGFP, 426GAL-α-syn-DsRed, 426GAL-α-syn-HA, 426GAL-α-syn-TAP and 426GAL-α-syn. These plasmids were transformed into the BY4741 yeast strain. To induce expression of the α-syn fusion proteins, yeast cells were pre-grown overnight in synthetic media containing raffinose to mid-log phase. The cells were switched to synthetic media containing galactose and grown for 6 h, then were either processed for fluorescence microscopy or protein lysates prepared for immunoblotting. Antibodies used for immunoblotting were: GFP (Roche, 1 : 1000); HA (Roche, 1 : 1000); TAP (Open Biosystems, 1 : 1000). Horseradish peroxidase-coupled secondary antibodies were used at 1 : 10000. For growth assays, serial dilutions of transformants were grown on solid synthetic media containing either glucose (control, α-syn 'off') or galactose (α-syn 'on').

Results and discussion

Overview of the Gateway system of recombination-based cloning

The types of plasmids and the general strategy of Gateway cloning is diagrammed in Figure 1A. This rapid, directional, and highly efficient cloning system is based on site-specific recombination mediated by the recombination machinery of bacteriophage lambda (Landy, 1989) and has been adapted for *in vitro* use in standard molecular biology protocols (Hartley *et al*., 2000; Walhout *et al*., 2000).

There are two main types of Gateway reactions: LR and BP. An LR reaction consists of an 'entry clone' plasmid (containing a gene of interest flanked by attL1 and attL2 sequences) harbouring a kanamycin resistance cassette that is mixed with a 'destination vector' (containing a bacterial death gene and chloramphenicol resistance gene flanked by attR1 and attR2 sequences), harbouring an ampicillin resistance cassette. LR Clonase II enzyme mix (Invitrogen) catalyses recombination between the recognition sites, generating an 'expression clone' containing the gene of interest in the destination vector backbone. Transformation of *E. coli* with this reaction mixture and plating

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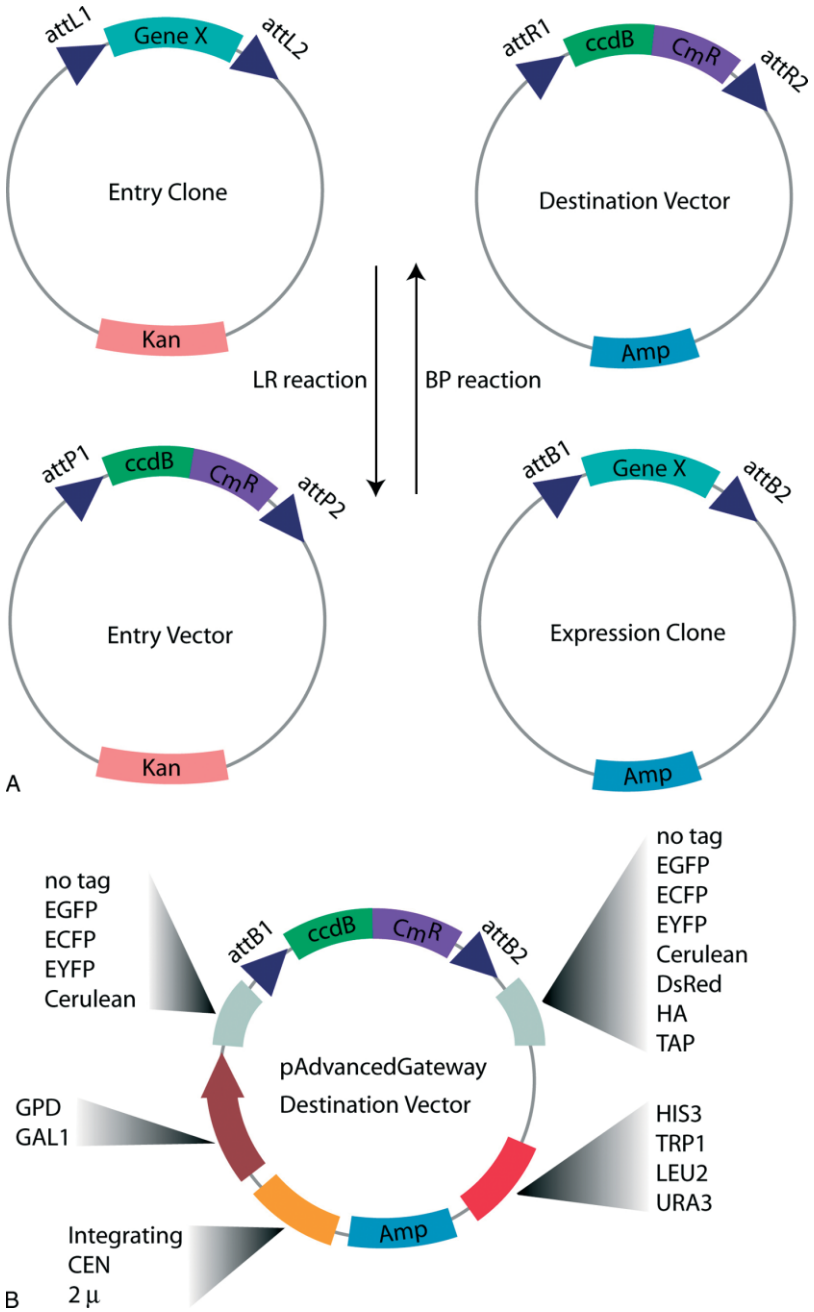

Figure 1. Overview of Gateway® system and generation of pAG yeast destination vectors. (A) The Gateway system of recombination-based cloning involves an LR reaction, in which an entry clone, containing a gene of interest is mixed with a destination vector, containing features of interest (e.g. promoter, protein tags, etc.). The destination vector harbours a recombination site-flanked bacterial 'death' gene (ccdB), which is exchanged for the gene of interest contained in the entry clone. Transformation of *E. coli*, which are sensitive to the ccdB effects, allows for selection of expression clones. This reaction is reversible (BP reaction); expression clones can be used to regenerate entry clones. (B) The set of pAdvancedGateway destination vectors allows for a choice of constitutive (GPD) or inducible (GAL1) promoters, N- or C-terminal protein tags, integrating or extra-chromosomal origins of replication, high- (2) or low-copy number (CEN), as well as a choice of auxotrophic markers. *CAUTION*: since these destination vectors contain the ccdB cassette, they must be propagated in ccdB-resistant *E. coli* (e.g. DB3.1)

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on ampicillin-containing LB agar plates allows for the specific selection of expression clones and the selective killing of bacteria containing the initial plasmid constructs. A BP reaction is essentially the opposite reaction of LR, and transfers genes from expression clones into entry vectors (isolated by selection on kanamycin-containing agar plates).

Construction and validation of a collection of *Saccharomyces cerevisiae* Gateway destination vectors

We modified the pRS series of yeast shuttle vectors (Christianson *et al*., 1992; Sikorski and Hieter, 1989) for use with the Gateway system (Figure 1B). This involved blunt-end ligation of the

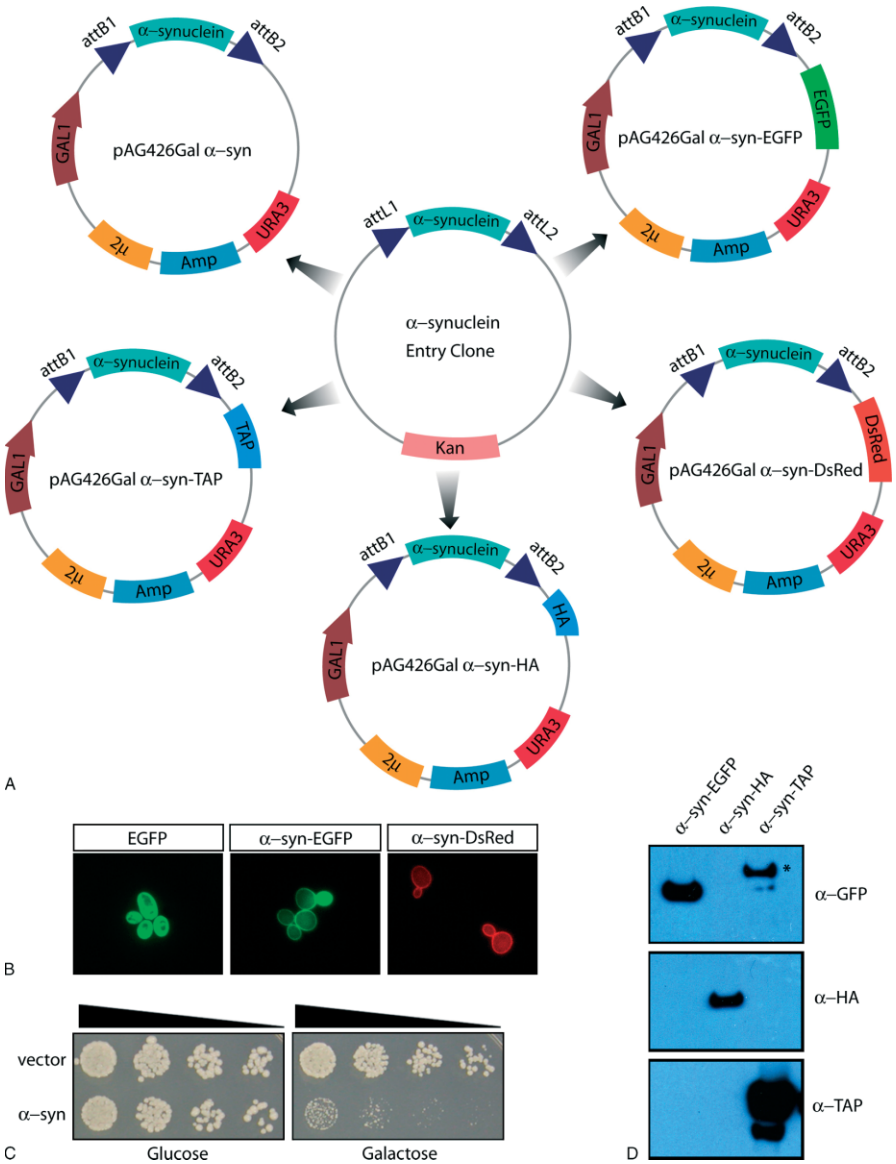

Figure 2. Practical application of Advanced Gateway vectors to experiments with α-synuclein in yeast. (A) An entry clone containing human α-synuclein (with or without stop codon) was used in 1 h *in vitro* LR reactions to generate untagged, EGFP-, DsRed-, HA- or TAP-tagged expression constructs. The Gateway-generated fluorescently-tagged proteins localized properly to the plasma membrane (B), whereas EGFP alone was distributed throughout the cytoplasm; the untagged protein induced cellular toxicity (C) and immunoblot analysis detected the appropriately sized tagged proteins (D). The asterisk (*) in (D) indicates a non-specific immunoreactive band resulting from the protein A portion of the TAP tag

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ccdB/chloramphenical resistance cassette into the multiple cloning site of each of these plasmids (see supplementary Table 1 for details on the type of plasmids generated). Diagnostic restriction digest and DNA sequencing verified proper orientation of the Gateway cassette. These initial Gateway vectors were subsequently modified to obtain a choice of N- or C-terminal protein tags to enable visualization by fluorescence microscopy or biochemical purification. We maintained the same nomenclature as the pRS series vectors, but renamed the new Advanced Gateway® derivatives, pAG (e.g. pAG413GPD, pAG426GAL, pAG306GAL-ccdB-EGFP, pAG413GAL-EGFP-ccdB). The ccdB name portion in plasmids for N- or C-terminal fusions stands for the entire Gateway cassette and is used in our nomenclature scheme to indicate the orientation of the Gateway cassette relative to the tag.

We tested the functionality of our vectors using a protein that we have been studying in our laboratory. The subcellular localization and phenotypic consequences of expressing human α-synuclein (α-syn) in yeast cells have been well characterized (Cooper *et al*., 2006; Outeiro and Lindquist, 2003). We used the Gateway system and our collection of pAG destination vectors to rapidly generate a variety of α-syn expression constructs, which we then tested in yeast cells. In a 1 h LR reaction we were able to generate a panel of α-syn constructs enabling toxicity studies, biochemical purification and subcellular localization (Figure 2). The generated α-syn fusion proteins behave according to previously published data, indicating that the linkers (see Table 3 for linker sequences) do not interfere with proper folding and targeting of α-syn. Generating a similar set of constructs using standard molecular biology techniques would take several days and would require customized cloning strategies for each construct. As with any fusion protein, the linker sequence, in certain contexts, potentially could affect the protein's function.

Table 3. Resulting linker sequences[a]

Primer	Sequence
EGFP, ECFP, EYFP, DsRed, Cerulean (C-terminal)	SAFLYKVVMGCRNSISSL*M*
TAP (C-terminal)	SAFLYKVVMGCRNSISSLSI GGPGGG*M*
3HA (C-terminal)	SAFLYKVVMGCRNSISSLSIGG*M*
EGFP, ECFP, EYFP, Cerulean (N-terminal)	GGGPGGGHQTSLYKKAE

[a] The translated attB1 or attB2 portion is underlined.

Concluding remarks

In summary, we have generated a collection of 288 *Saccharomyces cerevisiae* Gateway vectors enabling the rapid and efficient generation of a variety of expression constructs. All of these vectors are available to the research community via Addgene (**http://www.addgene.org/yeast_gateway**), individually or as an entire 'kit', and will complement the collection of existing yeast Gateway vectors (Geiser, 2005; Van Mullem *et al*., 2003). Further versions of the Gateway-based vectors we generate will be made available through Addgene.

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References

Christianson TW, Sikorski RS, Dante M, Shero JH, Hieter P. 1992. Multifunctional yeast high-copy-number shuttle vectors. *Gene* **110**: 119–122.

Cooper AA, Gitler AD, Cashikar A, *et al*. 2006. α-Synuclein blocks ER–Golgi traffic and Rab1 rescues neuron loss in Parkinson's models. *Science* **313**: 324–328.

Geiser JR. 2005. Recombinational cloning vectors for regulated expression in *Saccharomyces cerevisiae*. *Biotechniques* **38**: 378, 382.

Hartley JL, Temple GF, Brasch MA. 2000. DNA cloning using *in vitro* site-specific recombination. *Genome Res* **10**: 1788–1795.

Landy A. 1989. Dynamic, structural, and regulatory aspects of lambda site-specific recombination. *Annu Rev Biochem* **58**: 913–949.

Outeiro TF, Lindquist S. 2003. Yeast cells provide insight into α-synuclein biology and pathobiology. *Science* **302**: 1772–1775.

Rizzo MA, Springer GH, Granada B, Piston DW. 2004. An improved cyan fluorescent protein variant useful for FRET. *Nat Biotechnol* **22**: 445–449.

Sikorski RS, Hieter P. 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. *Genetics* **122**: 19–27.

Sopko R, Huang D, Preston N, *et al*. 2006. Mapping pathways and phenotypes by systematic gene overexpression. *Mol Cell* **21**: 319–330.

Van Mullem V, Wery M, De Bolle X, Vandenhaute J. 2003. Construction of a set of *Saccharomyces cerevisiae* vectors designed for recombinational cloning. *Yeast* **20**: 739–746.

Walhout AJ, Temple GF, Brasch MA, *et al*. 2000. Gateway recombinational cloning: application to the cloning of large numbers of open reading frames or ORFeomes. *Methods Enzymol* **328**: 575–592.

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