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Yeast Functional Analysis Report

A versatile toolbox for PCR-based tagging of yeast genes: new fluorescent proteins, more markers and promoter substitution cassettes

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Abstract

Tagging of genes by chromosomal integration of PCR amplified cassettes is a widely used and fast method to label proteins *in vivo* in the yeast *Saccharomyces cerevisiae*. This strategy directs the amplified tags to the desired chromosomal loci due to flanking homologous sequences provided by the PCR-primers, thus enabling the selective introduction of any sequence at any place of a gene, e.g. for the generation of C-terminal tagged genes or for the exchange of the promoter and N-terminal tagging of a gene. To make this method most powerful we constructed a series of 76 novel cassettes, containing a broad variety of C-terminal epitope tags as well as nine different promoter substitutions in combination with N-terminal tags. Furthermore, new selection markers have been introduced. The tags include the so far brightest and most yeast-optimized version of the red fluorescent protein, called RedStar2, as well as all other commonly used fluorescent proteins and tags used for the detection and purification of proteins and protein complexes. Using the provided cassettes for N- and C-terminal gene tagging or for deletion of any given gene, a set of only four primers is required, which makes this method very cost-effective and reproducible. This new toolbox should help to speed up the analysis of gene function in yeast, on the level of single genes, as well as in systematic approaches. Copyright © 2004 John Wiley & Sons, Ltd.

Introduction

The targeted introduction of heterologous DNA to genomic locations by a simple polymerase chain reaction (PCR)-based strategy has been widely used for research, particularly with the fungi *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* (Bahler *et al*., 1998; Baudin *et al*., 1993; Knop *et al*., 1999; Krawchuk and Wahls, 1999; Longtine *et al*., 1998; Schneider *et al*., 1995; Tasto *et al*., 2001; Wach *et al*., 1994, 1997). These strategies have been shown to be powerful tools in systematic gene deletion, protein localization and protein complex purification (Gavin *et al*., 2002; Ho *et al*., 2002), as well as for single gene-function analysis. The strategy requires: (a) a pair of primers that contain within their 5′ region sequences of homology to the genomic target location; and (b) PCR-cassettes (also termed 'modules') that can be amplified using these primers. To make the technique most powerful and cost-efficient, we constructed a series of new cassettes and included in all of them identical primer-binding sites, which allow the amplification of all C-terminal tags with only one pair of primers per gene. An additional primer is needed for gene deletion (Knop *et al*., 1999) and

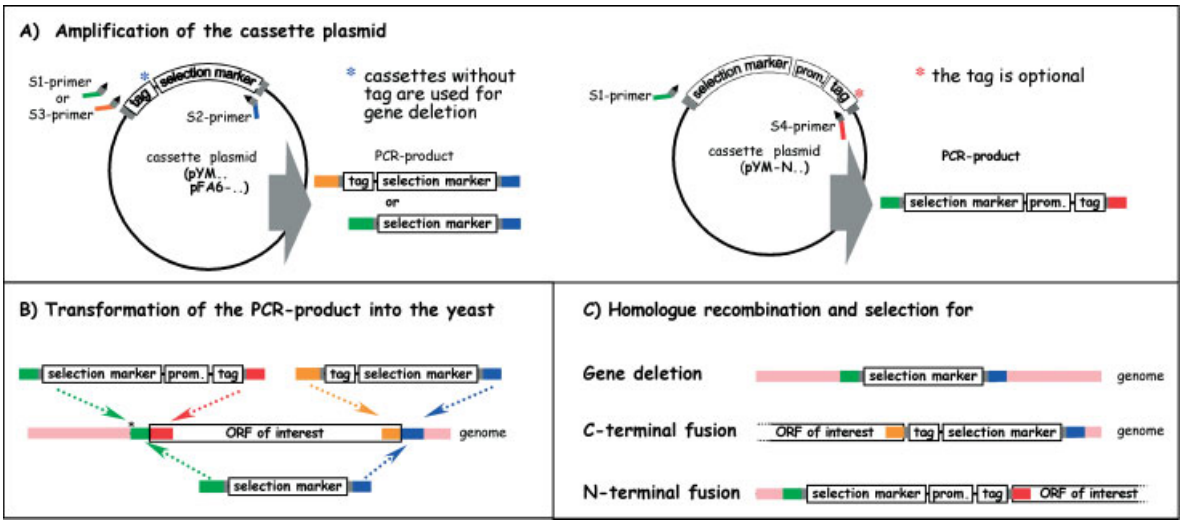

Figure 1. The principle of PCR-based epitope tagging. Schematic illustration of the principle of genomic manipulation of yeast strains using PCR-based strategies. The plasmid contains a cassette, which consists of a selection marker and additional sequences, which can be promoter sequences and/or sequences that encode for a tag (e.g. GFP). The S1-, S2-, S3- and S4-primers allow amplification of cassettes (A) and targeting of the respective PCR product to the desired genomic location (B), which becomes defined by the overhangs provided by the S1- S2-, S3- and S4-primers (see colour-encoded primers in the figure: the same colours indicate homologous sequences). Depending on whether a gene deletion, a C- or a N-terminal gene fusion should be performed, specific pairs of the S1- S2-, S3- or S4-primers are used to amplify the cassette. Upon transformation, an integration of the cassettes into the yeast genome occurs due to homologous recombination (C). For primer designs, see Figure 2

a fourth primer for the introduction of sequences at the N-terminus (Figure 1).

In addition to the previously published 12 cassettes for C-terminal epitope tags (Knop *et al.*, 1999), we present here a wider range of C-terminal tags as well as two new selection markers, both carrying dominant antibiotic-resistance genes. We also describe new cassettes that allow the replacement of the promoter of a given gene, with the optional addition of an N-terminal epitope tag to the gene. Nine promoters, five of them inducible, were cloned into different cassette plasmids.

The construction of PCR-cassettes is straightforward and can be done via standard cloning strategies (details provided upon request). Therefore, it will be easy to create new cassettes, e.g. to introduce new combinations of tags, makers and promoters (in the case of N-terminal tagging) by simple cloning procedures.

Materials and methods

Cassette plasmid construction

Standard techniques were used for DNA manipulations (Sambrook *et al.*, 1989). The construction of the PCR-cassette pYM1-12 is described in Knop *et al.* (1999). The construction of the new cassettes is summarized in Table 1; the primers used are listed in Table 2 (further details can be obtained upon request). A comprehensive overview of all available C-terminal tagging cassettes, with regard to selection marker and tag, is provided in Table 3.

Amplification of the PCR-modules

A set of four primers allows to amplify all N- and C-terminal tags and to generate gene deletions. The principle of the primer design is explained in Figure 2. The amplification of the modules can cause problems, because the annealing sites for S1, S2 and S3 primers (Figure 2), which were chosen initially for the EUROFAN project, lead to self-annealing of the primers. Another problem is the high GC content of the *natNT2* marker. To circumvent these problems, different PCR conditions have been used (Goldstein and McCusker, 1999). We present here one particular condition, which works well in several laboratories. One other reason for the failure of the PCR

Table 1. Properties and construction of the new cassette plasmids

Name	Used with primers	Size of product	Pro-moter[1]	Tag	Marker	Primers[2]	Template/ origin of tag or promoter	Target plasmid	Restriction sites used	Control digest
pFA6a–natNT2	S1/S2-	1460		—	*natNT2*	*natMX4-1/natMX4-2*[3]	*pEG202*[4]	*pFA6–natMX4*[7,3]	*Xho*I/*Sac*I	*Not*I 2390+1394 bp
pFA6a–hphNT1	S1/S2-	1840		—	*hphNT1*	*ADH1-1/ADH1-2 hphMX4-1/hphMX4-2*[5]	*p425-Gal1*[6]	*pFA6–hphMX4*[5,7]	*Xho*I/*Sac*I	*Not*I 2390+1777 bp
pYM13	S2/S3-	2330		*TAP*	*kanMX4*	*M13-hin/CYC1-Term CBP-s/CBP-as*	Oligos annealed	*pYM8*	*Sal*I/*Bam*HI	*Sal*I/*Xba*I 4568+111 bp
pYM14	S2/S3-	1820		*6HA*	*kanMX4*	*pYM3/6_F/pYM3/6_R*	*pYM3*	*pFA6a–kanMX4*	*Sal*I/*Bgl*II c/o *Bam*HI	*Not*I 2390+1782 bp *Hin*dIII/*Xho*I 2955+1217 bp
pYM15	S2/S3-	1670		*6HA*	*HIS3MX6*	*pYM3/6_F/pYM3/6_R*	*pYM3*	*pFA6a–HisMX6*	*Sal*I/*Bgl*II c/o *Bam*HI	*Not*I 2390+1626 bp
pYM16	S2/S3-	2050		*6HA*	*hphNT1*	*pYM3/6_F/pYM3/6_R*	*pYM3*	*pKS133*	*Sal*I/*Bgl*II c/o *Bam*HI	*Not*I 2390+2011 bp
pYM17	S2/S3-	1670		*6HA*	*natNT2*	*pYM3/6_F/pYM3/6_R*	*pYM3*	*pKS134*	*Sal*I/*Bgl*II c/o *Bam*HI	*Not*I 2390+1628 bp *Hin*dIII/*Xho*I 2767+1251 bp
pYM18	S2/S3-	1990		*9Myc*	*kanMX4*	*pYM3/6_F/pYM3/6_R*	*pYM6*	*pFA6a–kanMX4*	*Sal*I/*Bgl*II c/o *Bam*HI	*Eco*RI/*Sal*I 2459+1881 bp
pYM19	S2/S3-	1830		*9Myc*	*HIS3MX6*	*pYM3/6_F/pYM3/6_R*	*pYM6*	*pFA6a–HISMX6*	*Sal*I/*Bgl*II c/o *Bam*HI	*Hin*dIII/*Pvu*I 3278+906 bp
pYM20	S2/S3-	2220		*9Myc*	*hphNT1*	*pYM3/6_F/pYM3/6_R*	*pYM6*	*pKS133*	*Sal*I/*Bgl*II c/o *Bam*HI	*Hin*dIII/*Xho*I 2697+1872 bp
pYM21	S2/S3-	1840		*9Myc*	*natNT2*	*pYM3/6_F/pYM3/6_R*	*pYM6*	*pKS134*	*Sal*I/*Bgl*II c/o *Bam*HI	*Hin*dIII/*Xho*I 2767+1419 bp
pYM22	S2/S3-	1310		*3HA*	*klTRP1*	*klTRP1-1/klTRP1-2*	*pYM3*	*pYM1*	*Bss*HII/*Eco*RI	*Not*I 2390+1268 bp
pYM23	S2/S3-	1330		*3Myc*	*klTRP1*	*klTRP1-1/klTRP1-2*	*pYM3*	*pYM4*	*Bss*HII/*Eco*RI	*Bam*HI/*Xho*I 2479+1200 bp
pYM24	S2/S3-	1910		*3HA*	*hphNT1*	No PCR	*pYM1*	*pKS133*	*Sal*I/*Bss*HII	*Not*I 2390+1875 bp *Bss*HII/*Sal*I 4141+124 bp
pYM25	S2/S3-	2550		*yeGFP*[8]	*hphNT1*	No PCR	*pYM12*	*pKS133*	*Sal*I/*Bss*HII	*Bam*HI/*Xho*I 2721+2184 bp *Bss*HII/*Sal*I 4141+764 bp
pYM26	S2/S3-	1950		*yeGFP*[8]	*klTRP1*	*klTRP1-1/klTRP1-2*	*pYM3*	*pYM12*	*Bss*HII/*Eco*RI	*Xho*I/*Xba*I 3710+588 bp
pYM27	S2/S3-	2550		*EGFP*	*kanMX4*	*GFP-4/GFP-6*	*pEGFP*[9]	*pYM1*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 4187+753 bp
pYM28	S2/S3-	2400		*EGFP*	*HIS3MX6*	*GFP-4/GFP-6*	*pEGFP*[9]	*pYM2*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 4831+753 bp
pYM29	S2/S3-	1950		*EGFP*	*klTRP1*	*GFP-4/GFP-6*	*pEGFP*[9]	*pYM3*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 3570+753 bp
pYM30	S2/S3-	2550		*ECFP*	*kanMX4*	*GFP-4/GFP-6*	*pECFP*[9]	*pYM1*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 4187+753 bp
pYM31	S2/S3-	2400		*ECFP*	*HIS3MX6*	*GFP-4/GFP-6*	*pECFP*[9]	*pYM2*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 4031+753 bp
pYM32	S2/S3-	1950		*ECFP*	*klTRP1*	*GFP-4/GFP-6*	*pECFP*[9]	*pYM3*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 3570+753 bp
pYM33	S2/S3-	2550		*EBFP*	*kanMX4*	*GFP-4/GFP-6*	*pEBFP*[9]	*pYM1*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 4187+753 bp
pYM34	S2/S3-	1950		*EBFP*	*klTRP1*	*GFP-4/GFP-6*	*pEBFP*[9]	*pYM1*	*Sal*I/*Bam*HI	*Bgl*II/*Eco*RI 3500+1446 bp
pYM35	S2/S3-	2520		*DsRed1*	*kanMX4*	*Red1-1/Red1-2*	*pDsRed1-N1*	*pYM4*	*Bss*HII/*Bam*HI	*Sal*I/*Stu*I 4391+475 bp
pYM36	S2/S3-	2000		*DsRed1*	*klTRP1*	No PCR	*pSM822*	*pSM825*	*Bss*HII/*Bam*HI	*Hin*dIII/*Xho*I 2455+1804 bp
pYM37	S2/S3-	2520		*DsRed*	*kanMX4*	*dsRED-1/dsRED-2*	*DsRed*[10]	*pFA6a–kanMX4*	*Bss*HII/*Bam*HI	*Not*I 2390+2251 bp
pYM38	S2/S3-	2520		*RedStar*	*kanMX4*	*dsRED-2/dsRED-7*	*RedStar*[10]	*pFA6a–kanMX4*	*Bss*HII/*Bam*HI	*Sal*I/*Nco*I 3521+1120 bp *Bss*HII/*Sal*I 3915+1186 bp

Table 1. Continued

Name	Used with primers	Size of product	Pro-moter[1]	Tag	Marker	Primers[2]	Template/ origin of tag or promoter	Target plasmid	Restriction sites used	Control digest
pYM39	S2/S3-	2600		*EYFP*	*kanMX4*	*GFP-4/GFP-6*	*pEYFP*[9]	*pYM1*	*Sal*I/*Bam*HI	*Sal*I/*Bam*HI 4187+753 bp
pYM40	S2/S3-	2820		*EYFP*	*hphNT1*	No PCR	*pYM-YK*	*pKS133*	*Sal*I/*Bss*HII	*Sal*I/*Xho*I 2715+2231 bp
pYM41	S2/S3-	2400		*EYFP*	*HIS3MX6*	*GFP-4/GFP-6*	*pEYFP*[9]	*pYM2*	*Sal*I/*Bam*HI	*Xba*I/*Pvu*I 2732+2058 bp
pYM42	S2/S3-	2150		*RedStar**[11]	*natNT2*	*RedStar2-BamHI/RedStar2-SalI*	*RedStar*[10]	*pKS134–1*	*Sal*I/*Bam*HI	*Bam*HI/*Sal*I 3778+717 bp
pYM43	S2/S3-	2150		*RedStar2*[15]	*natNT2*	Site-directed mutagenesis[15]	*RedStar2*	*pKS134–1*	*Sal*I/*Bam*HI	*Bam*HI/*Sal*I 3778+717 bp
pYM44	S2/S3-	2310		*yeGFP*[8]	*HIS3MX6*	No PCR	*pYM5*	*pYM12*	*Bst*EII/*Eco*RI	*Sal*I/*Bss*HII 4141+763 bp
pYM45	S2/S3-	1740		*1HA*	*kanMX4*	*HA-F1/HA-F2*	Oligos annealed	*pYM1*	*Sal*I/*Bss*HII	*Bgl*II/*Eco*RI 2730+1446 bp
pYM46	S2/S3-	1760		*1Myc–7His*	*kanMX4*	*MYC-7xHis-F1/MYC-7xHis-F2*	Oligos annealed	*pYM1*	*Sal*I/*Bss*HII	*Bgl*II/*Eco*RI 2488+1446+266 bp
pYM47	S2/S3-	1852		*FlAsH*	*hphNT1*	*FlAsH-1/FlAsH-2*	Oligos annealed	*pYM–hphNT1*	*Sal*I/*Bam*HI	*Bam*HI/*Xho*I 2751+1446 bp
pYM48	S2/S3-	2569		*PA–GFP*[12]	*hphNT1*	*GFP-4/GFP-6*	*PA-GFP*	*pYM–hphNT1*	*Sal*I/*Bam*HI	*Bam*HI/*Xho*I 3468+1446 bp
pYM51	S2/S3-	2500		*eqFP611*[13]	*KanMX4*	*eqFP611-1/-2*	*pBS-KS+eqFP611*[13]	*pYM12*	*Sal*I/*Bss*HII	*Bam*HI/*Xho*I 4184+704
pYM–N1	S1/S4-	1990	*CUP1-1*	—	*kanMX4*	*CUP1-A/CUP1-B*	Yeast genomic DNA	*PFA6a–kanMX4*	*Sac*I/*Eco*RI	*Not*I 2390+2031 bp
pYM–N2	S1/S4-	1830	*CUP1-1*	—	*natNT2*	*CUP1-A/CUP1-B*	Yeast genomic DNA	*pYM–natNT2*	*Sac*I/*Eco*RI	*Not*I 2390+1874 bp *Hin*dIII/*Xho*I 3247+1017 bp
pYM–N3	S1/S4-	1980	*CUP1-1*	*3HA*	*natNT2*	*HA-1%CUP/HA-2%CUP*	*pYM1*	*pMM40*	*Bsp*EI/*Eco*RI	*Sal*I/*Xba*I 2848+1564 bp
pYM–N4	S1/S4-	2590	*CUP1-1*	*yeGFP*[8]	*natNT2*	*eGFP%CUP-1/eGFP%CUP-2*	*pYM12*	*pMM40*	*Bsp*EI/*Eco*RI	*Sal*I/*Xba*I 3454+1564 bp
pYM–N5	S1/S4-	2260	*CUP1-1*	*ProA*	*natNT2*	*ProA-1n/ProA-2n*	*pCW804*	*pMM40*	*Bsp*EI/*Eco*RI	*Sal*I/*Xba*I 3127+1564 bp
pYM–N6	S1/S4-	2987	*ADH*	—	*kanMX4*	No PCR	*p413-ADH*[14]	*pYM–N1*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 1480 bp
pYM–N7	S1/S4-	2827	*ADH*	—	*natNT2*	No PCR	*p413-ADH*[14]	*pYM–N2*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 1480 bp
pYM–N8	S1/S4-	2977	*ADH*	*3HA*	*natNT2*	No PCR	*p413-ADH*[14]	*pYM–N3*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 1628 bp
pYM–N9	S1/S4-	3587	*ADH*	*yeGFP*[8]	*natNT2*	No PCR	*p413-ADH*[14]	*pYM–N4*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 2234 bp
pYM–N10	S1/S4-	1816	*CYC1*	—	*kanMX4*	No PCR	*p413-CYC1*[14]	*pYM–N1*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 309 bp
pYM–N11	S1/S4-	1656	*CYC1*	—	*natNT2*	No PCR	*p413-CYC1*[14]	*pYM–N2*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 309 bp
pYM–N12	S1/S4-	1806	*CYC1*	*3HA*	*natNT2*	No PCR	*p413-CYC1*[14]	*pYM–N3*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 457 bp
pYM–N13	S1/S4-	2416	*CYC1*	*yeGFP*[8]	*natNT2*	No PCR	*p413-CYC1*[14]	*pYM–N4*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 1063 bp
pYM–N14	S1/S4-	2143	*GPD*	—	*kanMX4*	No PCR	*p413-GPD*[14]	*pYM–N1*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 639 bp
pYM–N15	S1/S4-	1983	*GPD*	—	*natNT2*	No PCR	*p413-GPD*[14]	*pYM–N2*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 639 bp
pYM–N16	S1/S4-	2133	*GPD*	*3HA*	*natNT2*	No PCR	*p413-GPD*[14]	*pYM–N3*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 784 bp
pYM–N17	S1/S4-	2743	*GPD*	*yeGFP*[8]	*natNT2*	No PCR	*p413-GPD*[14]	*pYM–N4*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 1390 bp
pYM–N18	S1/S4-	1932	*TEF*	—	*kanMX4*	No PCR	*p413-TEF*[14]	*pYM–N1*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 425 bp
pYM–N19	S1/S4-	1772	*TEF*	—	*natNT2*	No PCR	*p413-TEF*[14]	*pYM–N2*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 425 bp
pYM–N20	S1/S4-	1922	*TEF*	*3HA*	*natNT2*	No PCR	*p413-TEF*[14]	*pYM–N3*	*Sac*I/*Sma*I c/o *Bsp*EI+Klenow	*Sac*I/*Eco*RI 573 bp

pYM–N21	S1/S4-	2532	*TEF*	*yeGFP*[8]	*natNT2*	No PCR	*p413-TEF*[14]	*pYM–N4*	*SacI/SmaI c/o BsplE+Klenow*	*SacI/EcoRI* 1179 bp
pYM–N22	S1/S4-	1978	*GAL1*	—	*kanMX4*	No PCR	*p413-GAL1*[6]	*pYM–N1*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 471 bp
pYM–N23	S1/S4-	1818	*GAL1*	—	*natNT2*	No PCR	*p413-GAL1*[6]	*pYM–N2*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 471 bp
pYM–N24	S1/S4-	1968	*GAL1*	*3HA*	*natNT2*	No PCR	*p413-GAL1*[6]	*pYM–N3*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 619 bp
pYM–N25	S1/S4-	2578	*GAL1*	*yeGFP*[8]	*natNT2*	No PCR	*p413-GAL1*[6]	*pYM–N4*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 1225 bp
pYM–N26	S1/S4-	1951	*GALL*	—	*kanMX4*	No PCR	*p413-GALL*[6]	*pYM–N1*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 444 bp
pYM–N27	S1/S4-	1791	*GALL*	—	*natNT2*	No PCR	*p413-GALL*[6]	*pYM–N2*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 444 bp
pYM–N28	S1/S4-	1941	*GALL*	*3HA*	*natNT2*	No PCR	*p413-GALL*[6]	*pYM–N3*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 592 bp
pYM–N29	S1/S4-	2551	*GALL*	*yeGFP*[8]	*natNT2*	No PCR	*p413-GALL*[6]	*pYM–N4*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 1198 bp
pYM–N30	S1/S4-	1935	*GALS*	—	*kanMX4*	No PCR	*p413-GALS*[6]	*pYM–N1*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 428 bp
pYM–N31	S1/S4-	1775	*GALS*	—	*natNT2*	No PCR	*p413-GALS*[6]	*pYM–N2*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 428 bp
pYM–N32	S1/S4-	1925	*GALS*	*3HA*	*natNT2*	No PCR	*p413-GALS*[6]	*pYM–N3*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 576 bp
pYM–N33	S1/S4-	2535	*GALS*	*yeGFP*[8]	*natNT2*	No PCR	*p413-GALS*[6]	*pYM–N4*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 1182 bp
pYM–N34	S1/S4-	1902	*MET25*	—	*kanMX4*	No PCR	*p413-MET25*[6]	*pYM–N1*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 395 bp
pYM–N35	S1/S4-	1742	*MET25*	—	*natNT2*	No PCR	*p413-MET25*[6]	*pYM–N2*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 395 bp
pYM–N36	S1/S4-	1892	*MET25*	*3HA*	*natNT2*	No PCR	*p413-MET25*[6]	*pYM–N3*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 543 bp
pYM–N37	S1/S4-	2505	*MET25*	*yeGFP*[8]	*natNT2*	No PCR	*p413-MET25*[6]	*pYM–N4*	*SacI/SmaI c/o BspEI+Klenow*	*SacI/EcoRI* 1149 bp

The four rightmost columns list the primers, plasmids and restriction sites used for PCR construction of the cassettes.

[1] For N-terminal tags.

[2] See Table 2 for primer sequences.

[3] Before subcloning of the ADH1-terminator, a *Xho*I site was introduced into plasmid *pFA6–natMX4* using the indicated primers and the Quickchange Kit (Clonetech).

[4] Gyuris *et al.*, 1993.

[5] Before subcloning of the CYC1-terminator, a XhoI site was introduced into plasmid pFA6-hphMX4 using the indicated primers and the Quickchange Kit (Clonetech).

[6] Mumberg *et al.*, 1995.

[7] Goldstein and McCusker, 1999.

[8] Cormack *et al.*, 1996.

[9] From Clonetech.

[10] Knop *et al.*, 2002.

[11] RedStar* is identical to RedStar except that the T217A mutation is missing, which causes an increase in green fluorescence (Bevis and Glick, 2002).

[12] Patterson and Lippincott-Schwartz, 2002.

[13] Wiedenmann *et al.*, 2002.

[14] Mumberg *et al.*, 1994.

[15] RedStar2 has been constructed by introduction of the T4 mutations (Bevis and Glick, 2002) into RedStar.

Table 2. Primer sequences

Primer name	Sequence (5′ → 3′)
ADH1-1	GACAGAGAGCTCGATTACAACAGGTGTTGTCCTC
ADH1-2	CTGGCCTCGAGGCGAATTTCTTATGATTTATGATTT
CBP-as	TCGACGCTAGCAGTAGTTGGAATATCATAATCAAGTGCCCCGGAGGATGAGATTTTCTTAAAGCGGTTGGCT GCTGAGACGGCTATGAAATTCTTTTTCCATCTTCTCTTG
CBP-s	TCGACAAGAGAAGATGGAAAAAGAATTTCATAGCCGTCTCAGCAGCCAACCGCTTTAAGAAAATCTCATCC TCCGGGGCACTTGATTATGATATTCCAACTACTGCTAGCG
CUP1-A	GCGACGGAGCTCTAGTAAGCCGATCCCATTACC
CUP1-B	CGACGAATTCTCTGTCGTCCGGATTTATGTGATGATTGATTGATTGATTG
CYC1-term	GACAGAGAGCTCGTTAAAGCCTTCGAGCGTCCC
dsRED-1	CGGGATCCGGAGCAGGTGCTGGTGCTGGTGCTGGAGCAATTCTGGGTAGATCTTCTAAGAACGTC
dsRED-2	AAGTGGCGCGCTTACAAGAACAAGTGGTGTCTAC
dsRED-7	CGGGATCCGGAGCAGGTGCTGGTGCTGGTGCTGGAGCAATTCTGAGTAGATCTTCTAAGAACGTC
eGFP%CUP1-1	GCACGACTCCGGAATGTCTAAAGGTGAAGAATTATTCAC
eGFP%CUP1-2	CATCCGAGAATTCTCTGTCGGACCAGCACCGGCACCGGCACCAGCACCGGCACCAGCACCTTTGTACAATT CATCCATACCATG
GFP-4	CCTGGGATCCTTACTTGTACAGCTCGTCCATGC
GFP-6	GCACTGGTCGACGGAGCAGGTGCTGGTGCTGGTGCTGGAGCAATGAGCAAGGGCGAGGAGC
HA-1%CUP	GCACGACTCCGGAATGGGTTACCCATACGATGTTCCTGACTATGCG
HA-2%CUP	CATCCGAGAATTCTCTGTCGGACCAGCACCGGCACCGGCACCAGCACCGGCACCAGCACCAGAGCACTGA GCAGCGTAATCT
HA-F1	TCGACTACCCATACGACGTCCCAGACTACGCTTAG
HA-F2	CGCGCTAAGCGTAGTCTGGGACGTCGTATGGGTAG
hphMX4-1	GGCAAAGGAATAATCTCGAGTACTGACAATAAAAAG
hphMX4-2	CTTTTTATTGTCAGTACTCGAGATTATTCCTTTGCC
klTRP1-1	AGTCTAGGCGCGCAAAGTGGAACGATCATTCAC
klTRP1-2	AGGCCGAATTCGAGCTCGCCTCGAGGC
M13hin	AGCGGATAACAATTTCACACAGGA
MYC-7xHis-F1	TCGACGAGCAGAAGCTGATTAGCGAGGAAGATCTGCACCACCATCACCATCACCATTAG
MYC-7xHis-F2	CGCGCTAATGGTGATGGTGATGGTGGTGCAGATCTTCCTCGCTAATCAGCTTCTGCTCG
natMX4-1	GCCCTGCCCCTAATCTCGAGTACTGACAATAAAAAG
natMX4-2	CTTTTTATTGTCAGTACTCGAGATTAGGGGCAGGGC
pYM3/6_F	AGCTTCGTACGCTGCAGGTCG
pYM3/6_R	GGTAAGATCTCTTGAATGATCGTTCCACTTTTTAGC
ProA-1n	GCACGACTCCGGAATGGCGCAACACGATGAAGCCGTAG
ProA-2n	CATCCGAGAATTCTCTGTCGGACCAGCACCGGCACCAGGAGCACCAGCGCCTGGAGCACCAGCACCATTCG CGTCTACTTTCGGCG
Red1-1	GGATCCGGAGCAGGTGCTGGTGCTGGTGCTGGAGCAATGGTGCGCTCCTCCAAGAACGTC
Red1-2	AGAAGTGGCGCGCAGCTACAGGAACAGGTGGTGGCGGCC
RedStar2-*Bam*HI	GCGAGGATCCTTACAAGAACAAGTGGTGTCTAC
RedStar2-*Sal*I	GGACACAGTCGACGGAGCTGGAGCTGGTGCAGGTGCTGGTGCAATGAGTGCTTCTTCTGAAGATGTCATCA CTGAATTCATGAGATTCAAG
FlAsH-1	TCGACTGTTGTCCAGGTTGTTGTGCTAGAGCCTGAG
FlAsH-2	GATCCTCAGGCTCTAGCACAACAACCTGGACAACAG
S2-SPC42	TACACAGAACGCTTTAAGAATGCGCCATACTCCTTAACTGCTTTTTAAATCATCAATCGATGAATTCGAGCTCG
S3-SPC42	CAAGCCTGAAAATAATATGTCAGAAACATTCGCAACTCCCACTCCCAATAATCGACGTACGCTGCAGGTCGAC
S2-SPC72	AGAGAGTGACTGAGTGTTACATTAAATATATTTATATATAAACGTATGATATTTAATCGATGAATTCGAGCTCG
S3-SPC72	ACAGGAAAATGAGTCATTGAGATCGAAACTTTTCAACCTATCAATCAACAATCCCCGTACGCTGCAGGTCGAC
S1-SSP1	TCACAATAGTGCCTATTATCATGATAGAAGTAGAGTAGAAAAGCTAGCAACAATGCGTACGCTGCAGGTCGAC
S4-SSP1	GGGAAGTTGAGGTTATTTCCCCAGAAGGATCATTCTCATATGTGCCAGAGCTTCTCATCGATGAA TTCTCTGTCG
S1-DON1	TATCTACTTGACTTTGGCTGGTATTTAAACACAAGTAAGAGAAGCATCAAACATGCGTACGCTGCAGGTCGAC
S4-DON1	TTAGAAAAGAGGTTTTAGCAGCATTATTTTCTTTTCCCTTTCTATTTTTCTTTCCCATCGATGAATTCTCTGTCG
eqFP611-1	GCAGCAGCAGCGCGCCTCGAGTCAAAGACGTCCCAGTTTG
eqFP611-2	GCGCAGCGCGGTCGACGGAGCAGGTGCTGGTGCTGGTGCTGGAGCAGGGATCCGTATGAATTCACTGATC AAGGAA

Table 3. Systematic table of all available pYM plasmids for C-terminal tagging and deletion

Tag	*kanMX4*	*hphNT1*	*natNT2*	*HIS3MX6*	*klTRP1*
Deletion module (no tag)	pFA6a–*kanMX4*	pYM–*hphNT1*	pYM–*natNT2*	pFA6a–*HIS3MX6*	
1HA	pYM45				
3HA	pYM1	pYM24		pYM2	pYM22
6HA	pYM14	pYM16	pYM17	pYM15	pYM3
1MYC–7His	pYM46				
3MYC	pYM4			pYM5	pYM23
9MYC	pYM18	pYM20	pYM21	pYM19	pYM6
ProA	pYM7				
TEV–ProA	pYM8				
TEV–ProA–7His	pYM9			pYM10	
TEV–GST–6HIS	pYM11				
TAP	pYM13				
yeGFP (em507, ex488 nm)	pYM12	pYM25		pYM44	pYM26
EGFP (em507, ex488 nm)	pYM27			pYM28	pYM29
ECFP (em475 (501), ex433 (453) nm)	pYM30			pYM31	pYM32
EBFP (em447, ex383 nm)[#]	pYM33				pYM34
DsRed1 (em583, ex558 nm)	pYM35				pYM36
DsRed (yRFP) (em583, ex558 nm)	pYM37				
RedStar (em583, ex558 nm)	pYM38				
RedStar* (em583, ex558 nm)	pYM42				
RedStar2 (em583, ex558 nm)	pYM43				
EYFP (em527, ex513 nm)	pYM39	pYM40		pYM41	
PA–GFP (photo activated GFP)		pYM48			
FlAsH		pYM47			
eqFP611 (em611, ex559 nm)	pYM51				

[#] BFP is a very weak fluorescent protein. So far, we have not yet successfully used the BFP-modules. However, we provide the cassette since some strongly expressed proteins might be well detected when tagged with BFP.

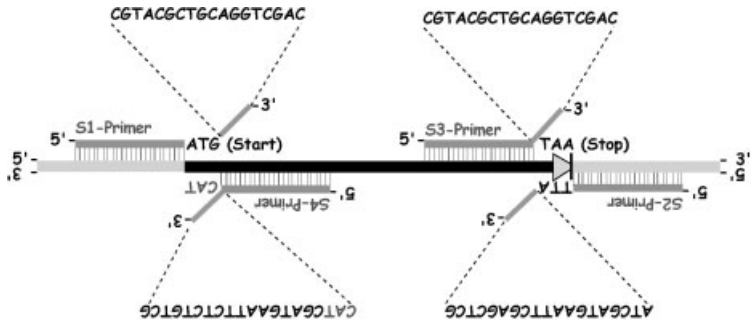

Figure 2. Primer design. The figure illustrates the design of the primers S1- S2-, S3- and S4 that are used for the amplification of the cassettes described in this paper. The correct primer design is fundamental for the success of the PCR amplification and the correct targeting into the yeast genome. The following rules should help to design the primers using specific software such as DNA Strider: S1-primer, 45–55 bases upstream of the ATG (including ATG = start codon) of the gene, followed by 5′-CGTACGCTGCAGGTCGAC-3′; S2-primer, the *reverse complement* of 45–55 bases downstream of the STOP-codon including STOP) of the gene, followed by 5′-ATCGATGAATTCGAGCTCG-3′; S3-primer, 45–55 bases before the STOP-codon (excluding STOP) of the gene, followed by 5′-CGTACGCTGCAGGTCGAC-3′; S4-primer, the *reverse complement* of 45–55 bases downstream of the ATG (start-codon) of the gene (excluding ATG), followed by 5′-CATCGATGAATTCTCTGTCG-3′

is often linked to the quality of the primers (see Discussion).

The pipetting scheme for a 50 µl reaction and the PCR cycle scheme are visualized in Figure 3A/B. A successful PCR gives a very strong band at the estimated size (Table 1, Figure 3C), when 3–5 µl of the PCR were analysed on a standard agarose gel. Some *natNT2* cassettes might cause problems. The use of another PCR-buffer (Figure 3C) circumvents this problem.

For transformation of S288c- or W303-derived strains, usually 5 µl of a PCR were used. For some other strain backgrounds (such as SK-1), a 10-fold higher amount of DNA was used. For this purpose, the PCR product was ethanol-precipitated and dissolved in water (1/10 of the original volume).

Yeast strains and growth conditions

YPD and synthetic drop-out media were prepared as described (Sherman, 1991). For antibiotic selection markers, the following concentrations of antibiotics were added to standard YPD-plates

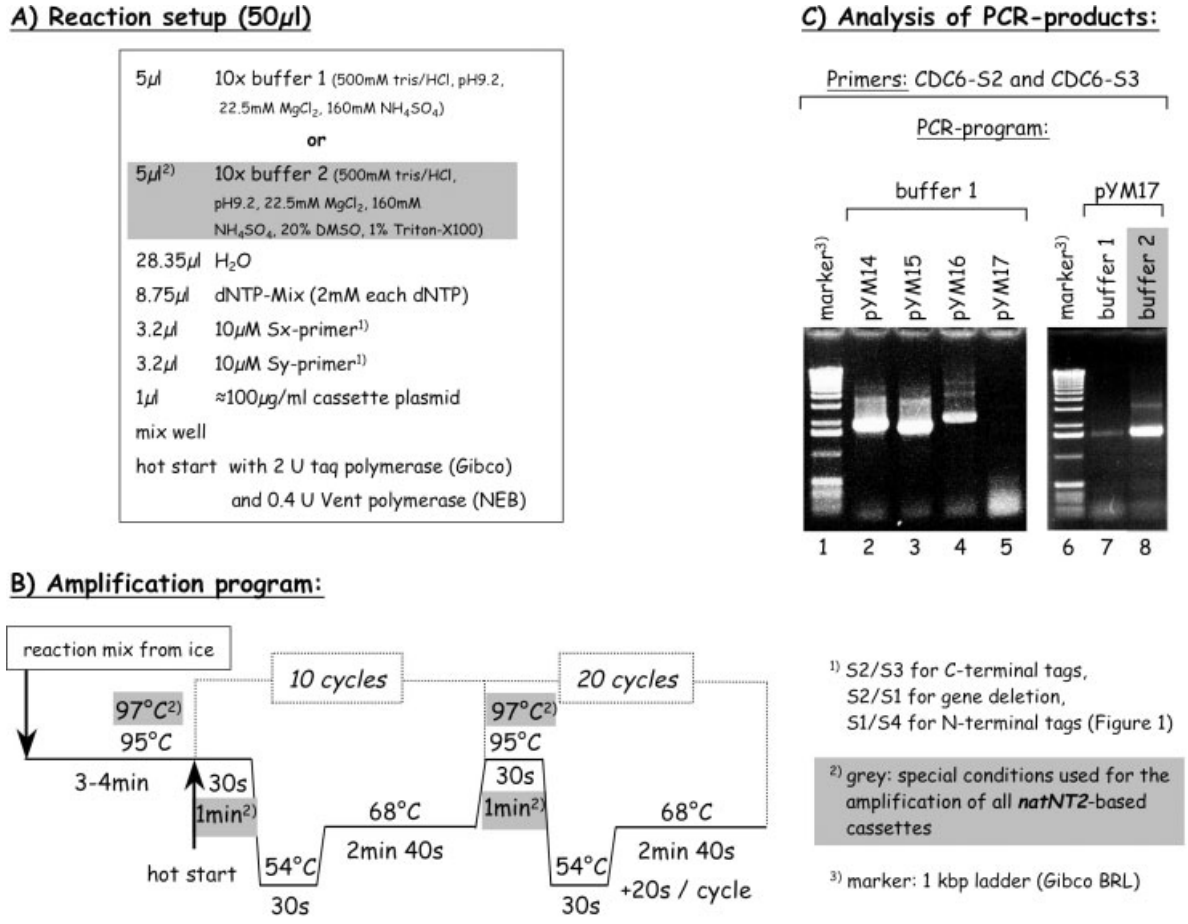

Figure 3. Amplification of PCR-cassettes. (A) 50 µl of a PCR-sample are mixed on ice. For the amplification of *hphNT1*- and *natNT2*-containing cassettes, it is recommended to use buffer 2. (B) The amplification programme is the same for all cassettes except the modification in the melting step for *natNT2*-based cassettes (grey-shaded). (C) pYM14-17 (6HA-tag) were amplified with the S2/S3 primers of *CDC6*. 5 µl of the PCR reaction were analysed on a 0.9% agarose-TAE gel. The gel was stained with ethidium bromide. As reference, 10 µl 1 kbp marker, diluted according to the manufacturers' instructions (Invitrogen, Gibco, BRL) was run (1, 6). Under standard conditions, amplification of pYM14 (2) and pYM15 (3) gave a very strong band at the expected size (Table 1). The amplification of pYM16 (4) was less efficient, but sufficient for transformation of the PCR-product; pYM17 could not be amplified under standard conditions (5). With the special protocol (B), pYM17 was weakly amplified in buffer 1 (7); a very strong PCR-product of the correct size (Table 1) was amplified when special conditions (B) and buffer 2 were used (8)

(**www.duke.edu/web/microlabs/mccusker/**; Goldstein and McCusker, 1999): *kanMX4*, geneticin (G418, GibcoBRL), 200 mg/l; *hphNT1*, hygromycin B (Cayla, Toulouse, France; **www.cayla.com**), 300 mg/l; and *natNT2*, nourseothricin (ClonNAT, Werner BioAgents, Jena-Cospeda, Germany; **www.webioage.com**), 100 mg/l.

The antibiotics were added after autoclaving and cooling of the medium to approximately 60 °C. In the case of ClonNAT, a sterile filtered stock-solution was prepared prior to addition to the medium, while for geneticin and hygromycin B, the powder and the solution provided by the manufacturer were used directly.

Yeast transformation and testing

Yeast transformation using frozen competent cells was based on the LiOAc method (Schiestl and Gietz, 1989), however with several modifications. A detailed description of the method is given in Knop *et al*. (1999).

For *klTRP1* or *HIS3MX6* selection, after transformation cells were resuspended in 200 µl sterile PBS and plated directly onto plates containing synthetic medium lacking the respective amino acid (SC-HIS, SC-TRP; Sherman, 1991).

For *kanMX4, hphNT1, natNT2*-selection: after transformation, cells were resuspended in 3 ml of YPAD medium and incubated on a shaker for at least 5–6 h at 30 °C, than sedimented and plated onto the selection plates.

Selection for positive transformants on plates containing antibiotics often requires replica plating of the plate after 2 days at 30 °C, because of the high background of transiently transformed cells, which makes it difficult to recognize the correct integrants (Knop *et al*., 1999; Wach *et al*., 1997). The success of the integration was tested by colony PCR using a quick chromosomal DNA isolation procedure (Finley and Brent, 1995), immunoblotting or by immunofluorescence, as described previously (Knop *et al*., 1999). For immunoblotting, protein extraction was done using the NaOH/βME/TCA-protocol (Knop *et al*., 1999).

For the detection of epitope-tagged proteins, tag-specific antibodies were used: HA-tag, mouse monoclonal 12CA5 (Roche Boehringer-Mannheim), 16B12 (Babco); Myc-tag, mouse monoclonal 9E10 (Boehringer-Ingelheim); Protein A/TAP-tag, rabbit PAP (DAKO); Don1p, affinity purified rabbit anti-Don1p (Rabitsch *et al*., 2001); GFP, affinity-purified sheep anti-GFP. For ECL detection (Amersham), goat anti-mouse, -rabbit or -sheep secondary antibodies coupled to horseradish peroxidase (Jackson Immuno Research Laboratories) were used.

Plasmid requests

The full collection of plasmids and the sequence files will be made available for non-commercial recipients through EUROSCARF (**http://www.uni-frankfurt.de/fb15/mikro/euroscarf/index.html**). The plasmids have been prepared and tested carefully; however, we cannot guarantee that no error has been made. In case of problems, please do not contact any of the authors unless you are *absolutely* sure that the problem is associated with the plasmid (use positive controls!).

Results

Two new selection markers: *hphNT1* and *natNT2*

Recently, Goldstein and McCusker (1999) introduced three new dominant drug resistance cassettes that can be used in the yeast *S. cerevisiae*. The cassettes were constructed in analogy to the pFA6–*kanMX4* marker (Goldstein and McCusker, 1999; Wach *et al*., 1994), thus allowing the use of the established S1/S2-primer annealing sites (Wach *et al*., 1994; Knop *et al*., 1999) for amplification. The *hphMX4* and *natMX4* (Goldstein and McCusker, 1999) markers confer resistance to hygromycin B or clonNat (nourseothricin), respectively, and were cloned in-between the promoter and terminator of the *kanMX4* cassette (Wach *et al*., 1994). The homologous sequences flanking the different marker genes, however, lead to recombination between the markers, if the two markers are used simultaneously in the same yeast strain. To circumvent this problem, we exchanged the terminator of the *hphMX4* cassette and replaced it with the terminator of the *CYC1* gene. Similarly, we replaced the *natMX4* terminator with the *ADH1* terminator. The new cassettes were termed *hphNT1* and *natNT2*, respectively (NT = new terminator; Table 1). As demonstrated in a control experiment (not shown), *kanMX4, natNT2* and *hphNT1* completely failed to recombine with each other.

C-terminal tagging: fluorescent proteins

The availability of a variety of fluorescent proteins, such as yeGFP (Cormack *et al.*, 1997), EGFP, EBFP, ECFP, EYFP (**http://www.clontech.com/gfp/excitation.shtml**), DsRed (Matz *et al.*, 1999), hcRED (Gurskaya *et al.*, 2001) and RedStar, a much brighter version of DsRed (Knop *et al.*, 2002), consequently led to the construction of new cassettes. The coding regions of the six fluorophores were cloned into tagging cassettes preceded by a spacer sequence that codes for the peptide 'SGAGAGAGAGAIL'. This spacer peptide can facilitate the correct folding of the fluorescent proteins when coupled to the protein of interest (Miller and Lindow, 1997). Additionally, we provide a cassette containing the red fluorescent protein eqFP611 (Wiedenmann *et al.*, 2002).

The properties of some of the GFP derivatives are summarized in a review article (Tsien, 1998; for spectral properties, see also Table 3). All of them have been successfully used for applications in baker's yeast, such as *in vivo* double labelling and live cell imaging. The suitability of each of the individual fluorescent proteins for a specific experiment, however, has to be tested each time.

The red fluorescent protein DsRed has been limited to special application in yeast (Pereira *et al.*, 2001), since the formation of the red chromophore (Baird *et al.*, 2000) is not fast enough ($T_{1/2} \sim 24$ h) to allow the detection of *de novo* synthesized proteins in logarithmically growing cells. This has been partially solved by the construction of a much brighter variant, called RedStar (Knop *et al.*, 2002), or by a faster-maturing but less bright variant named T4-DsRed (Bevis and Glick, 2002). We constructed a combination of the T4-DsRed and the RedStar mutant, which leads to a bright, fast-maturing red fluorescent protein, RedStar2. We provide for several of these DsRed variants cassettes (Table 1), most of which contain yeast codon optimized constructs. The last drawback of DsRed-variants, their strong tetramerization (Baird *et al.*, 2000), has only recently been solved (Campbell *et al.*, 2002), but this monomeric DsRed variant seems to be not yet bright enough for general applications in yeast (unpublished observation). However, the red fluorescent protein eqFP611 (Wiedenmann *et al.*, 2002) largely circumvents this problem.

Double labelling using different fluorescent proteins

For double-fluorescent labelling, different fluorescent proteins can be combined: GFP together with DsRed, GFP and BFP, GFP and CFP, and YFP in conjunction with CFP. The combination of YFP and CFP is frequently used. The tagged proteins can be distinguished with appropriate filters. However, both, CFP and YFP bleach faster then GFP. The CFP signals often appeared weakly fluorescent when observed by eye; however, imaging with a CCD-camera gave nice and strong signals (Figure 4).

C-terminal tagging: HA, MYC and TAP tag

HA and MYC-tags are used for the detection of the tagged proteins by immunoblotting and immunofluorescence microscopy. A combination of two tagged proteins (HA and MYC, respectively) in one strain is widely used to detect protein–protein interaction by co-immunoprecipitation. Furthermore, it became obvious that proteins with low expression levels can be detected when several repeats of the HA or Myc tag (6HA or 9Myc) were fused to the protein. On the other hand, too many tags may interfere with the functionality of the fusion protein. For native protein purification, it has been shown that single HA-tagged proteins can be eluted from anti-HA beads using the HA peptide (YPYDVPDYA), while this was not possible when multiple tags were used. Because of these considerations, we constructed a variety of PCR modules using single, triple and hexa- or nona-tags in combination with a variety of selection markers (Table 3), thus enabling the flexible construction of strains carrying different tags at the same time.

The use of Protein A as an affinity tag has shown to be a powerful tool for the purification of proteins from yeast lysates, especially in combination with a calmodulin-binding peptide (CBP) and a TEV site-specific protease cleavage site. This combination of features, called the TAP tag (Rigaut *et al.*, 1999), has been shown to be very useful for native protein complex purification (Gavin *et al.*, 2002).

An example for the application of the TAP tag PCR module (pYM13) is shown in Figure 5.

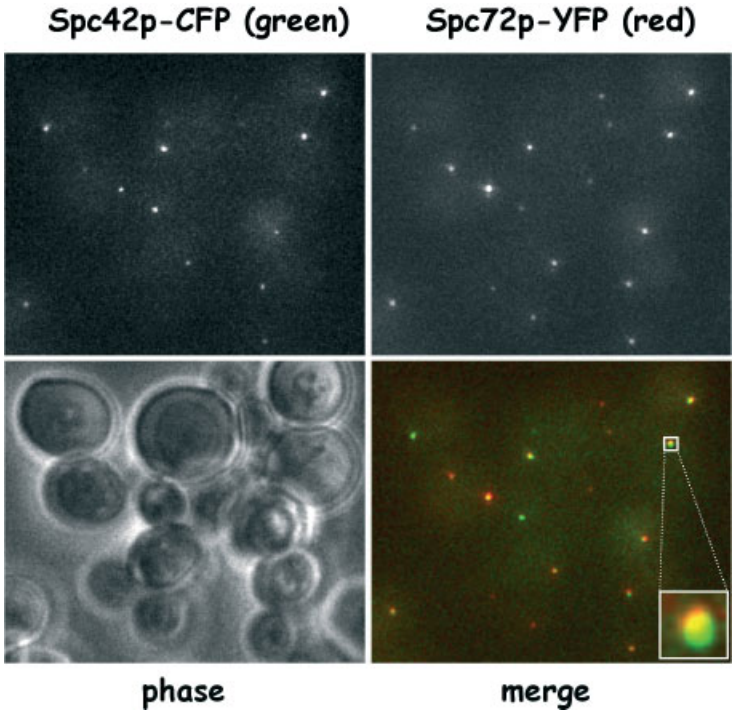

Figure 4. Double labelling of two C-terminal tagged proteins: CFP and YFP. *SPC42* was tagged with CFP amplified from the cassette plasmid pYM30; *SPC72* was tagged with YFP amplified from pYM41, using the corresponding S2 and S3 primers (Table 2). The cells were collected in logarithmic growth and fixed for 5 min with 4% (w/v) paraformaldehyde. The cells were analysed by fluorescence microscopy

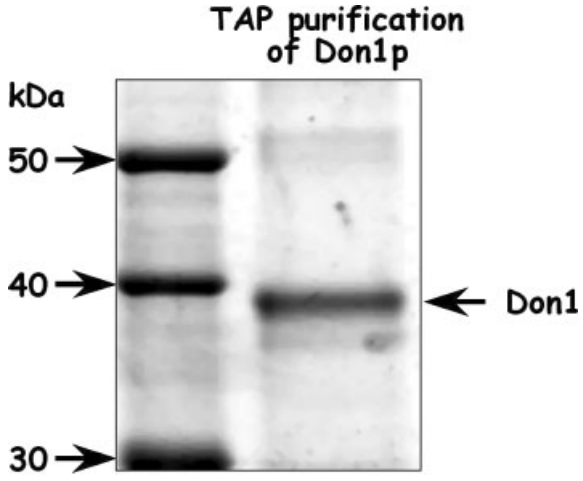

Figure 5. Purification of Don1p using the TAP-tag. The protein Don1p was tagged with the TAP tag using pYM13 and *DON1* specific S2- and S3-primers. The protein was purified from the soluble fraction of meiotic cells using a modified version of the protocol of Rigaut *et al.* (1999)

Other tags

Recently, other tags with specific properties became fashionable. The FlAsH tag consists of a small peptide, containing four cystein residues (DCCPGCCA), that is recognized by specific di-arsenic compounds, which, upon binding, become fluorescent (Adams *et al.*, 2001). We have tested the FlAsH tag and found that it worked also in yeast; however the maximally obtainable level of fluorescence, when compared with the analogous GFP fusion, was less than 5%, thus limiting the usefulness of this tag. Similarly, we also constructed a cassette containing the photo-activatable GFP (PA-GFP; Patterson and Lippincott-Schwartz, 2002). Proteins carrying this tag emitted, when maximally activated, less than 10% of the fluorescence compared to GFP-tagged versions. This limits the usefulness of this tag in yeast.

Promoter replacement and N-terminal tagging

The introduction of a heterologous promoter upstream of the START codon of a gene is a way

to control and to modulate gene expression. At the same time, it allows the introduction of a N-terminal epitope tag to the gene.

We constructed a set of cassettes with nine different replacement promoters. Eight of these promoters were well characterized from previous applications in centromeric or 2 μ plasmids (Mumberg *et al*., 1994, 1995). The replacement of an internal promoter with the constitutive *ADH, CYC1, GPD* or *TEF* promoters can be used to modulate the expression of a gene in a permanent manner. For inducible expression, the *GAL1* promoter and two truncated (and weaker) derivatives of this promoter, termed *GALL* and *GALS* (Mumberg *et al*., 1994), as well as the *MET25* promoter, are provided. All the promoters were cloned into cassettes with *kanMX4* and *natNT2* selection markers. Additionally, all *natNT2* promoter-substitution cassettes were combined with a N-terminal 3HA and yeGFP (Cormack *et al*., 1997) tag (Table 1). We observed different expression rates of the gene *DON1* when controlled by the eight different promoters. The inducible promoters are not always completely repressed in the non-induced state. In the case of the relatively strong *MET25* and the *GAL1* promoters, a weak expression was observed in the repressed state of the promoter (glucose complete medium; Figure 6). In contrast, the two weaker versions of the *GAL*-promoter, *GALL* and *GALS*, were completely repressed (Figure 6).

Furthermore, we constructed five cassettes containing the *CUP1-1* promoter (Table 1). This strong promoter can be induced with $CuSO_4$. We used this system successfully for the regulated induction of gene expression during various phases of the meiotic cell cycle (unpublished data). An example of the expression of Ssp1p under control of *CUP1-1* is given in Figure 7.

Discussion

In the present paper, we describe 37 new cassettes for the C-terminal epitope tagging of yeast proteins, developed by combining existing tags with new marker genes and cloning new tags, namely a variety of different fluorescent proteins of all available colours, and the TAP-tag. Furthermore, a series of 37 N-terminal cassettes has been developed that allow, besides the replacement of the promoter of the target gene, the introduction of N-terminal tags. For one single gene, all these cassettes can be amplified with four unique primers (Figures 1 and 2). The versatility of the primers is a strong advantage not only regarding to the cost of the method. Also, once all four primers have been successfully tested, any concerns about the quality of the primers can be omitted, which can turn out in some cases to be quite important (see below). The cloning strategies for most of the cassettes were based on common restriction sites, which facilitate the construction of further cassettes, if necessary (Table 1; further details available upon request).

PCR amplification and primers

Since the PCR amplification of the cassettes has caused problems in different laboratories, we describe a PCR-protocol suitable for the amplification of almost all of the cassettes. This protocol works well (in several laboratories), and fulfils three major criteria: reliability, fidelity and high yield. It requires, however, a reliable PCR machine that allows time increment programming. For the amplification of *natNT2*-based cassettes, this protocol needs to be slightly modified due to the high GC content of the coding sequence of this marker gene (see Materials and methods; Figure 3). Another reason why sometimes the PCR does not work is the poor primer quality. We found, that for some suppliers, up to 20% of the primers do not work (e.g. 40% of the PCRs performed), while for other suppliers, less then 5% are non-functional (less than 10% of PCRs performed) with respect to amplification of modules. Testing the primers in combination with established primers can help to nail down the faulty primer (companies normally will provide a free replacement primer).

New selection markers

The use of the *hphNT1* and *natNT2* cassettes is as robust as the *kanMX4* cassettes. Cells selected on antibiotic media tend to form a lawn, due to the growth of transiently transformed cells, which might hinder the identification of positive clones. In such a case the cells were replica-plated after 2 days of growth onto a fresh plate of the same medium. On the new plate, only positive clones grow. Using *kanMX4* and *HIS3MX6* together in one strain led to recombination events within the

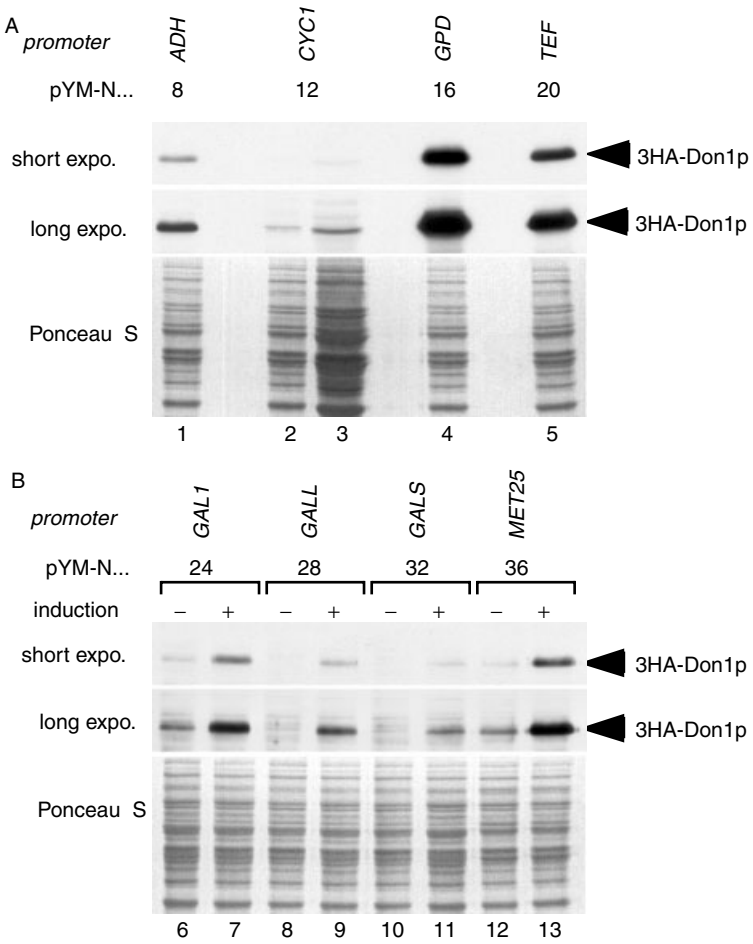

Figure 6. Control of expression of *DON1* using a range of different promoter substitutions. The promoter of the gene *DON1* was exchanged for all available promoters (except *CUP1-1*; cf. Figure 7) associated with the N-terminal 3HA-tag. Cultures were grown into the exponential growth phase. Western blot detection was done with the monoclonal antibody 16B12. Equal protein load was verified by staining the blots with Ponceau S. Two different expositions are shown to underline the differences in promoter strength. (A) Constitutive promoters: *GPD* (lane 4) and *TEF* (lane 5) induce very strong protein expression; the *ADH*-promoter (lane 1) is weaker; whereas the *CYC1*-promotor (lane 2) is very weak, therefore it was detected with a 5× protein load (lane 3); 12 μg (60 μg in lane 3) total protein were analysed. (B) Inducible promoters: induction was performed by adding 1% glucose (–) or 1% galactose (+) to YEP-raffinose medium (all *GAL*-promoters) or by washing and transferring the culture to SC-met medium (*MET25*-promoter). Induction time was 90 min. 12 μg total protein were analysed. The inducible promoters are different in strength; the very strong *MET25* and the strong *GAL1* are slightly leaky (lanes 6 and 12)

marker genes. After the transformation of the second cassette, positive clones must be selected on both, G418 and SC-His plates. The *klTRP1* cassette seems to promote a somewhat less-than-wild-type growth rate when used to complement the *trp1* mutation; therefore, it is recommended to wait 2 more days in case no colonies appear 2–3 days after transformation. Usually, transformants were confirmed using colony PCR in combination with either immunoblotting using anti-HA, anti-Myc, anti-GFP or PAP (for detection of protein A tags) antibodies or fluorescence microscopy (to visualize fusions with fluorescent proteins) or indirect immunofluorescence microscopy (HA or Myc fusions).

New fluorescent markers

We observed that yeGFP (Cormack *et al*., 1997) and EGFP (Clontech) do not show observable

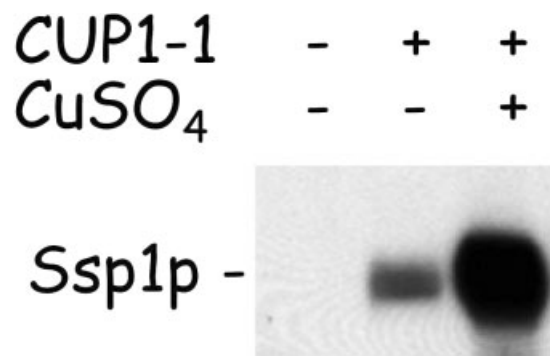

Figure 7. Control of expression of *SSP1* by the *CUP1-1* promoter. The gene *SSP1*, expressed only during meiosis (Moreno-Borchart *et al.*, 2001), was chromosomally tagged using pYM-N1 and S1 and S4 primers for *SSP1*. Expression of the gene was detected in mitotically growing cultures. Ssp1p expression was followed in the *CUP1-1-SSP1* strain and in a control strain with the unaltered *SSP1* gene in the absence or presence of $CuSO_4$ (100 μM, 2 h), as indicated in the figure. Upon cell lysis, Ssp1p was detected using a specific antibody

differences in brightness, although they do contain different mutations compared to the wild-type GFP.

We have also provided a number of different cassettes containing DsRed and mutagenized versions of DsRed. Due to the properties of the DsRed protein, its application is somewhat limited compared to GFP. This is mainly due to its strong tetramerization (Baird *et al.*, 2000), which can interfere with protein function (Knop *et al.*, 2002). Table 4 summarizes some of the properties of the different red fluorescent proteins that are contained in our cassettes.

Promoter exchange and N-terminal tagging

A new feature of the presented set of cassettes are the 37 new promoter substitution and N-terminal tagging modules. Apart from the *CUP1-1* promoter, which was specifically cloned for N-terminal tagging of proteins that are involved in meiosis, all other promoters were taken from existing yeast plasmids, therefore, their expression levels have already been studied in detail and the promoters can be used according to these data (Mumberg *et al.*, 1994, 1995). The promoter substitution can be applied for the determination of expression level-related phenomena, or simply to deplete a gene product. It was noted that, while the *GAL1* and the *MET25* promoters were slightly leaky under repressive conditions, the less active *GALL* and *GALS* were tightly repressed in glucose medium (unpublished data and Figure 6). The use of the *GALS* promoter might thus be a better tool than the until now frequently used *GAL1* promoter, first because of the reduced leakiness, but also for the lower expression rate in the induced state.

Conclusion

Taken together, the new range of PCR-cassettes allows the use of more selection markers, the combination of more tags in one single strain and the application of fluorescence double labelling with CFP and YFP, but also with GFP and RedStar2, while dsRed and RedStar can be used as fluorescent timers (see above and Table 4). N-terminal tags and promoter substitutions allow to interfere with transcriptional regulation and to conditionally deplete gene products, while the availability

Table 4. Properties of the red fluorescent protein

Name	Mutations	Tm1/2 (h)	Aggregation	Brightness relative to DsRed	Codon usage
DsRed		24	+++	1	Yeast
RedStar[1]	G2S, R18K, V97I, S113T, F125L, M183K, P187Q, T203I	Approx. 12	+	10–20[2]	Yeast
T4-DsRed[3]	R2A, K5E, N6D, T21S, H41D, N42Q, V44A, A145P, T217A	0.7	–	0.3[4]	Original
RedStar2[1]	Combination of RedStar$^+$ T4–DsRed mutations	Approx. 0.5–1[5]	–	2–4[5]	Yeast

[1] The yeast codon optimized sequence of DsRed, RedStar and RedStar2, contain an additional codon at position 2.
[2] Knop *et al.*, 2002.
[3] Please note that T4-DsRed is not included in the list of cassettes available.
[4] Bevis and Glick, 2002.
[5] Value not determined precisely.

of N-terminal tags provides the possibility to label proteins that cannot be tagged at the C-terminus. The need of only four different primers for the use of all cassettes described here and in Knop *et al*. (1999) makes the tagging cheap, reliable and flexible. However, the ease by which new strains can be constructed by this method should, of course, never prevent us from keeping one key question in mind: how does this manipulation affect the function of the gene?

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