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**Yeast Functional Analysis Report**

# Expanding the repertoire of plasmids for PCR-mediated epitope tagging in yeast

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## Abstract

**Epitope tagging of yeast proteins provides a convenient means of tracking proteins of interest in Western blots and immunoprecipitation experiments without the need to raise and test specific antibodies. We have constructed four plasmids for use as templates in PCR-based epitope tagging in the yeast *Saccharomyces cerevisiae*. These plasmids expand the range of epitopes available in a tag–*URA3*–tag context to include the FLAG, HSV, V5 and VSV-G epitopes. The cloning strategy used would be easily applicable to the construction of a similar tag–URA3–tag molecule for essentially any desired epitope. Oligonucleotides designed for PCR from one plasmid may be used interchangeably with any of the other template molecules to allow tagging with different epitopes without the need for new primer synthesis. We have tagged Tfc6 with each of the triple epitope tags and assessed the efficiency of these epitopes for chromatin immunoprecipitation (ChIP). For all the tagged alleles, ChIP occupancy signals are easily detectable at known Tfc6 target genes. These new tags provide additional options in experimental schemes requiring multiple tagged proteins. Copyright © 2008 John Wiley & Sons, Ltd.**

**Keywords: yeast; vectors; plasmids; epitope tagging; FLAG; HSV; V5; VSV-G; ChIP; chromatin immunoprecipitation**

## Introduction

The use of epitope-tagged proteins in Western blots and immunoprecipitation experiments obviates the need to raise and test specific antibodies, thus saving considerable time and expense. Antibodies against epitope tags are well characterized, of demonstrated quality and widely available. Frequently they are monoclonal antibodies, decreasing the possibility of batch-to-batch variability. It is often desirable to follow several different proteins in the context of a single strain, and to do this one must use a different epitope tag for each one. It is therefore useful to extend the range of molecules for tagging with various epitopes for which antibodies are readily available. This allows the creation of strains harbouring several different epitope-tagged proteins.

When substituting a gene at its normal locus with an allele encoding an epitope-tagged version of the protein, one would traditionally have first constructed a *URA3*-marked integrating plasmid containing the tagged allele and used this for two-step gene replacement (Guthrie and Fink, 1991). The entire plasmid would be linearized and integrated into the genome at the relevant locus, followed by selection for ura$^-$ recombinants on 5-fluoroorotic acid (5-FOA). This method required extra time for cloning steps, and the recombination could yield either the desired mutant strain or the original parental strain, necessitating the screening of large numbers of colonies to obtain the tagged strain.

In a slight variation on traditional two-step gene replacement, Alani *et al*. (1987) developed a disruption cassette consisting of the *URA3* gene flanked by two perfect repeats of a sequence. This

module would be cloned into the target gene on a plasmid, and the entire cassette plus flanking sequences sufficient for homologous recombination into the target locus would be excised and introduced into yeast. The perfectly repeated sequences would then recombine to remove the *URA3* gene, leaving one copy of the repeated sequence at the disruption site and reclaiming the *URA3* marker for the strain. The advantage of this method over traditional two-step strategies using integrating plasmids is that in this disruption-based method the parental strain cannot be regenerated by recombination; all loopouts should be the correct mutants.

Schneider *et al*. (1995) introduced a convenient PCR-based variant of this method to allow epitope tagging of any desired yeast protein. The pMPY plasmids described in that study serve as PCR templates for tagging with HA and MYC. Each consists of the *URA3* gene flanked by identical repeats of a sequence encoding three copies of either the myc or the HA epitope. This tag–*URA3*–tag sequence is used as a template for PCR primers designed with tails homologous to the genomic target locus. The PCR product is introduced into the desired yeast strain by transformation, and the tails of the primers target the tag–*URA3*–tag cassette to the region of interest. After integration, recombination occurs between the two perfectly identical tagging sequences, resulting in the removal of the *URA3* gene, such that only one copy of the tagging sequence remains at the site of integration.

Here we introduce four new plasmids for PCR-based modification of yeast genes at their normal genomic loci to generate variants encoding epitope-tagged proteins. The molecules are derived in part from the pMPY plasmids of Schneider *et al*. (1995) and are designed to be completely interchangeable and modular with those plasmids. For any given locus, only a single pair of gene-specific primers (the same as the primers used for pMPY-based tagging) need be designed to allow tagging with any of the epitopes.

## Materials and methods

### Plasmid construction

DNA fragments encoding triple copies of each of the four epitopes were generated by mutually primed synthesis with the following oligonucleotide pairs: for the triple FLAG epitope, STR12552 cctgaattcgagctcGACTATAAAGACGACGACGACAAAgcggcgGATTACAAGGATGATGATGATAAGgctgca and STR12553 CAGActcgagactagtCTTATCGTCATCGTCCTTATAATCtgcagcCTTATCATCATCATCC; for the triple VSV-G epitope, STR13468 cctgaattcgagctcTATACTGATATTGAAATGAATCGATTAGGTAAAgctgccTACACCGACATCGAGATGAACCGC and STR13469 cagactcgagactagtTTTTCCAAGTCGGTTCATTTCTATGTCTGTATAtgcggcCTTGCCCAAGCGGTTCATCTCGAT; for the triple HSV epitope, STR13470 cctgaattcgagctcCAACCTGAGCTAGCTCCTGAAGATCCCGAAGATgctgccCAGCCCGAGTTGGCACCCGAGGAC and STR13471 cagactcgagactagtGTCTTCTGGGTCTTCTGGTGCAAGCTCTGGTTGtgctgcGTCCTCTGGGTCCTCGGGTGCCAAC; for the triple V5 epitope, STR13472 cctgaattcgagctcGGTAAACCTATTCCTAATCCTCTCCTAGGTTTAGATTCTACTgctgccGGCAAGCCCATCCCCAACCCCTTGCTTGGCTTGGACT and STR13473 cagactcgagactagtTGTTGAATCAAGTCCGAGAAGTGGATTTGGTATTGGTTTTCCtgcggcGGTGGAGTCCAAGCCA.

Each double-stranded triple-epitope fragment was introduced into pBluescript SK as a *Sac*I–*Spe*I fragment. The resulting vector was digested with *Spe*I and *Eco*RI and ligated to the *Spe*I–*Eco*RI *URA3*-containing insert from pMPY-myc. Finally, a second copy of the triple-epitope fragment was introduced into this molecule as an *Eco*RI–*Xho*I fragment. Cloning was verified by DNA sequencing.

### Strain construction

For construction of epitope-tagged Tfc6 strains, primers STR12574 GTTATGCATTTTCTAATAGTGCTGGACTATTAACACTCGAATACCTGTCAagggaacaaaagctgg and STR12575 CAACAATAGTTCAATGTCACAAATTGTATTTATTACGTAAAGTCCATCTCActatagggcgaattgg were used in standard PCR reactions with Pfu DNA polymerase, using each of the new molecules as a template. PCR products were checked by agarose gel and then each used to transform yeast strain BY4742 (Brachmann *et al*., 1998). $Ura^+$ transformants were selected on medium lacking uracil and the colonies subjected to two rounds of purification on $ura^-$ plates before being streaked onto medium containing 5-FOA for selection of



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ura$^-$ recombinants. Recombinants were checked by colony PCR with oligos STR13191 (TAAGGCTTTGGAAATGGGATT) at position +1799 and STR13192 (CGTCAACAATAGTTCAATGTCAC) at +2070.

### Western blotting

Whole-cell extracts from each tagged strain and the untagged parental strain were separated by electrophoresis on a 10% polyacrylamide gel, which was blotted to nitrocellulose. The nitrocellulose filter was cut into pieces, each representing one tagged/untagged strain pair, and Western blotting was performed using antibodies obtained from Sigma-Aldrich (anti-FLAG Sigma #F3165; anti-HSV Sigma #H6030; anti-V5 Sigma #V8012; anti-VSV glycoprotein Sigma #V5507) at the dilutions recommended by the manufacturer. Secondary antibodies were obtained from Jackson ImmunoResearch and used at 1/10 000 dilution.

### Chromatin immunoprecipitation

ChIP was performed essentially as previously described (Kuras and Struhl, 1999; Mencia *et al*., 2002). Exponentially growing yeast strains were crosslinked with formaldehyde, and chromatin was isolated from each strain and subjected to immunoprecipitation with 2 μg of the relevant antibody. The amount of immunoprecipitated DNA from selected loci was measured using quantitative PCR in real time on an Applied Biosystems 7300 thermal cycler. Experiments were performed a minimum of twice.

## Results and discussion

We have constructed four plasmids modelled on the pMPY plasmids of Schneider *et al*. (1995). Each is designed to facilitate PCR-based tagging of yeast genes with DNA encoding three copies of a convenient epitope against which high-quality antibodies are commercially available. Each plasmid contains the *URA3* gene flanked by two identical stretches of DNA encoding three copies of a given epitope tag. The generalized sequence of the PCR template is shown in Figure 1A. The epitope tags represented by these plasmids are FLAG (DYKDDDDK; Hopp *et al*., 1988), HSV (QPELAPEDPED), V5 (GKPIPNPLLGLDST) and VSV-G (YTDIEMNRLGK). The sequences of the cassettes encoding each triple epitope tag are shown in Figure 1B. To minimize undesired recombination, the tagging sequences were chosen so as to minimize nucleotide sequence identity among the three repeats of each epitope. The tagging modules were also designed with identifying restriction sites, so as to be easily distinguishable from one another by restriction digestion. Details of the cloning strategy used to produce these molecules are given in the methods section.

To modify a gene of interest using these molecules, PCR primers must be designed to amplify from the template and have tails with sufficient identity to the target locus to allow homologous recombination. The sequences required for amplification from any of the new templates are identical to those used for PCR using pMPY templates; the same primer pair may thus be used for PCR tagging with any of the epitopes. The PCR product is transformed into the desired yeast strain, and integrants are selected on medium lacking uracil. The Ura$^+$ transformants are subjected to two rounds of purification by restreaking on to medium lacking uracil. These Ura$^+$ colonies are then streaked onto medium containing 5-FOA to select for ura$^-$ recombinants. The ura$^-$ recombinants are purified by restreaking to 5-FOA medium and then verified by PCR product size change after colony PCR.

As in the pMPY plasmids, a stop codon is present after the second repeat of the tagging sequence to ensure proper protein termination in the case of C-terminal tagging. If the molecules are used for N-terminal tagging, care must be taken to design the PCR primers to eliminate this stop codon. Figure 1C shows the sequence of primers needed for C-terminal tagging; the sequence of primers for N-terminal tagging is shown in Figure 1D. N-terminal tagging in haploid yeast strains using these templates is suitable only for genes nonessential for viability, because before the 5-FOA loopout step, the *URA3*-containing PCR product will intervene between the ORF and its promoter, effectively eliminating expression of the ORF. This problem may be circumvented by tagging one allele in a diploid strain, inducing sporulation, and then dissecting tetrads to obtain the desired haploid.

To test the new molecules, we performed PCR-mediated tagging of Tfc6, an integral component



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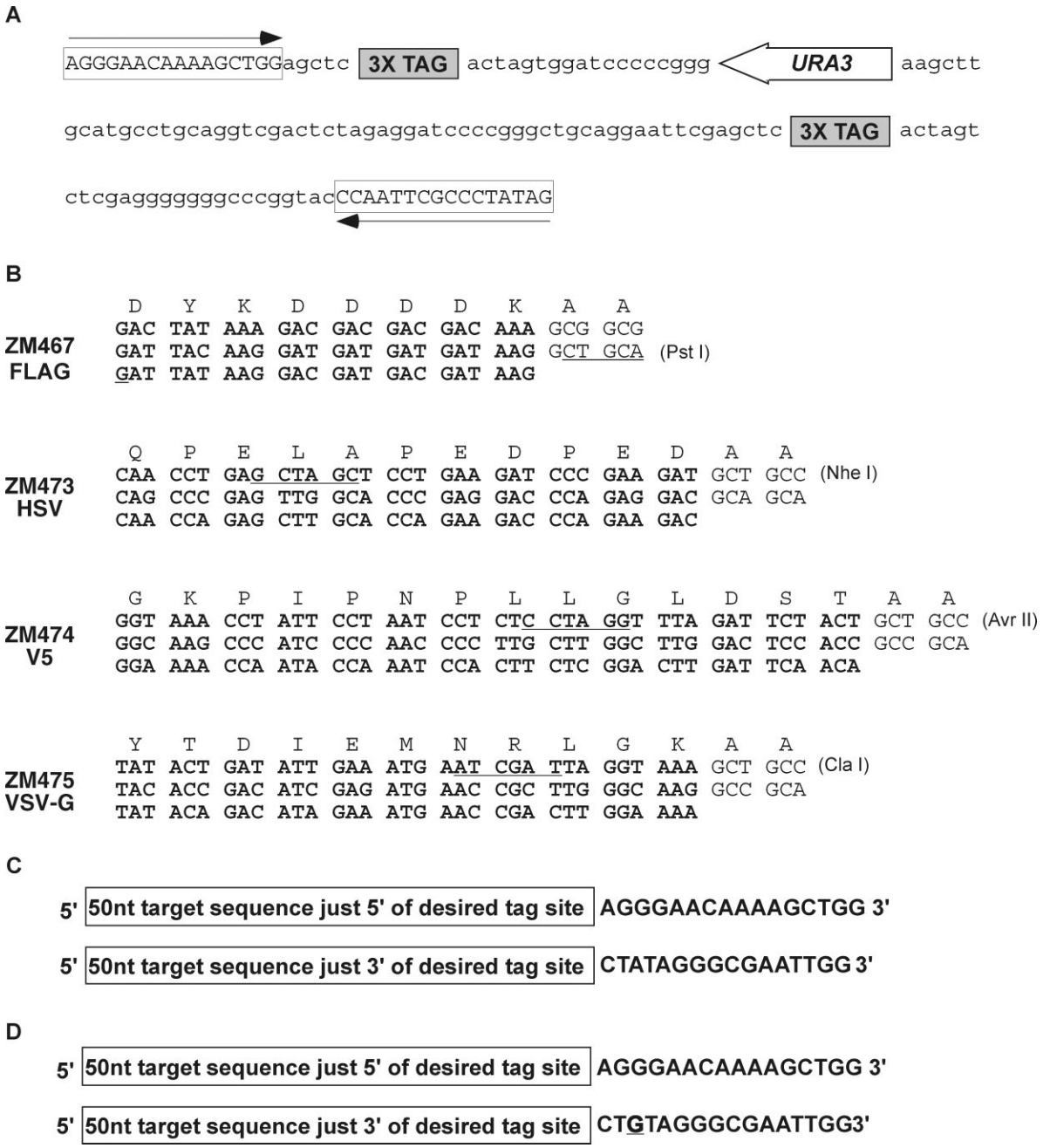


**Figure 1.** Vector design. (A) Generalized sequence of the PCR template in the new vectors. The boxed sequences at the ends indicate the sites of PCR primer annealing. The *URA3* arrow represents the sequence of the genomic *URA3* *Hin*dIII–*Sma*I fragment. (B) Sequences of the triple tag encoding regions from each of the four plasmids. Sequences in bold encode repeats of the epitope; in between each repeat are linker sequences encoding two alanine residues. The amino acid sequence is shown above the first repeat. The identifying restriction site for each triple tag sequence is underlined, with the restriction enzyme named in parentheses. (C) Sequence of the primers used for C-terminal PCR-based tagging. The boxed regions represent target-specific sequence for homologous recombination at the desired integration site. These primers introduce an in-frame stop codon and are therefore suitable for tagging only at the 3′ end of the ORF, just before the natural stop codon. (D) Sequences of the PCR primers needed for N-terminal PCR-based tagging. The underlining indicates a nucleotide change needed to remove the in-frame stop codon



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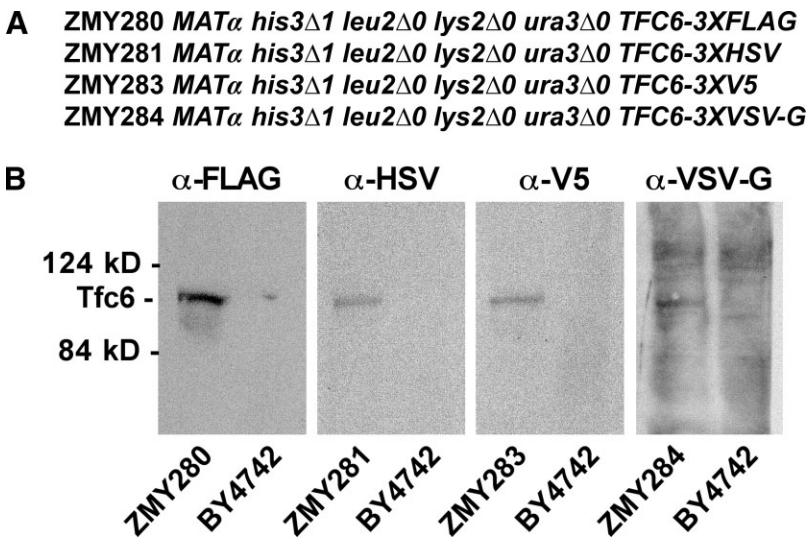


**Figure 2.** Expression of epitope-tagged proteins. (A) List of strains generated in this work. (B) Western blots showing the presence of tagged Tfc6 in each of the new strains. The left lane of each blot contains protein from the indicated epitope-tagged strain; the right lane contains protein from the untagged parent strain BY4742. The antibody used is indicated above each blot

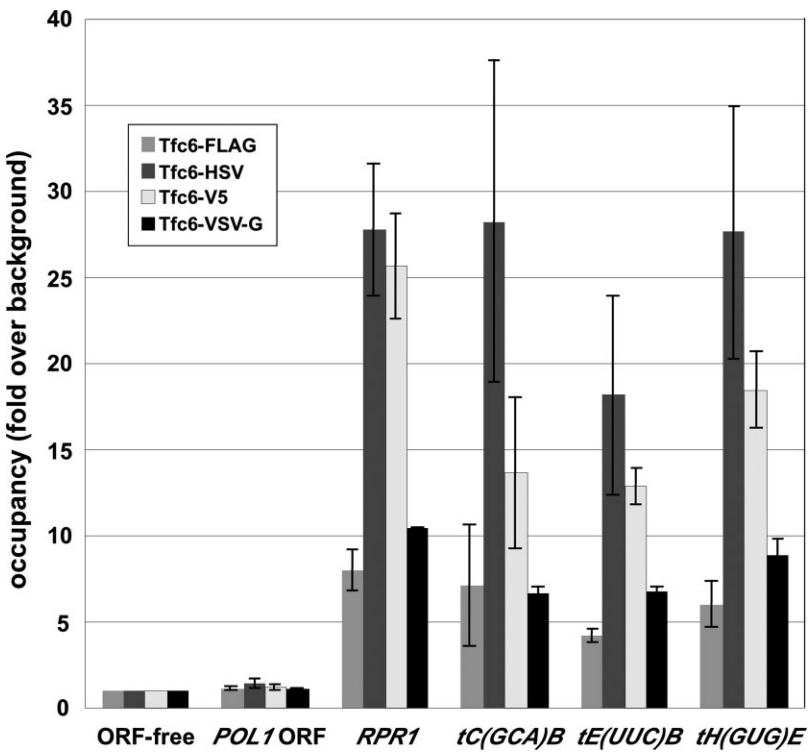


**Figure 3.** ChIP of Tfc6 from each of the tagged strains. For each strain, ChIP was performed using the relevant antibody. Real-time PCR was used to measure the amount of immunoprecipitated DNA at the indicated loci. All results are represented as fold over background, which is defined as immunoprecipitation efficiency at the negative control locus designated 'ORF-free'. *POL1* represents a locus in the middle of the open reading frame for the gene encoding the catalytic subunit of DNA polymerase I. As a Pol II-transcribed gene, this locus is not occupied by Tfc6 and is included as a negative control



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of the RNA Polymerase III transcription apparatus, at its C-terminus with each of the four tagging cassettes. The strain names are listed in Figure 2A. We verified the presence of epitope-tagged protein in each strain by Western blotting, using antibodies against the relevant epitope (Figure 2B). For all four strains, a band corresponding to tagged Tfc6 was detected in the tagged but not the parental strain. We noted non-specific background signal on the anti-VSV-G blot that was observed in two independent experiments, suggesting that this antibody cross-reacts with other components of the yeast extract.

Tfc6 is a subunit of the recognition factor TFIIC, which has been well characterized with respect to its chromatin immunoprecipitation (ChIP) occupancy (Harismendy *et al*., 2003; Moqtaderi and Struhl, 2004; Roberts *et al*., 2003). To compare the efficacies of the four epitope tags in ChIP experiments, we performed ChIP on the tagged Tfc6 strains. We easily detected Tfc6 occupancy at several of its known genomic targets, demonstrating that the new triple epitope tags are all useful for ChIP (Figure 3). The occupancy values obtained for the HSV and V5 epitopes were moderately (two- to four-fold) higher than those obtained for FLAG and VSV-G, which were comparable to results previously obtained for triply-myc-tagged Tfc6 (data not shown). We do not know whether for other tagged proteins the HSV and V5 epitopes would consistently yield higher signals. An untagged strain control yielded background values for all loci tested (data not shown).

The availability of several different tags in a modular format makes it easy to generate differently tagged versions of the same protein using a single pair of tagging primers. This flexibility can be useful when the strain into which one wishes to introduce a tagged protein already harbours one or more other tagged proteins. This allows the creation of strains in which, for example, several proteins involved in different stages of a given pathway are all tagged for easy immunological study. One caveat of using such strains is the risk that an epitope-tagged version of a protein might have some imperceptible functional impairment compared to the wild-type protein. In strains harbouring several tagged proteins, any mildly deleterious effects of the individual tagged proteins might cumulatively hamper cell growth.

Many antibodies against peptides are currently available from commercial sources. We note that it is possible by the cloning strategy used here to construct similar tag–*URA3*–tag plasmids for tagging with a great variety of corresponding epitopes.

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