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Full Length Research Paper

FLAG tag module for PCR based gene targeting

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Epitope tagging of yeast proteins has become an efficient tool for biochemical analysis of protein of interest. The epitope-tagged proteins can be used for western blotting, immunoprecipitation and immunofluorescence experiments without the need to raise specific antibodies, thus saving considerable time and expense. We have constructed plasmid containing FLAG tag with kanMX6 module, which allows selection of G418-resistant cells in yeast. The same set of primers that amplify module constructed by Bahler et al. (1998) can be used to amplify the FLAG tag module constructed in this study. The linear DNA fragment containing FLAG tag module with flanking homology region of gene of interest can be efficiently integrated on the yeast genome, using homologous recombination. We have successfully FLAG tag wat1/pop3 gene at its chromosomal locus and confirmed by western blot analysis. This construct can be very useful for generating C terminal tagging of desired genes at its normal chromosomal locus without interfering with their function.

Key words: *S. pombe*, epitope tagging, FLAG tag, pFA6a plasmid, *wat1/ pop3*.

INTRODUCTION

Protein tagging with in-frame sequences encoding epitope tag allows simple and efficient biochemical analysis of gene products (Smith and Johnson, 1988; Kolodziej and Young, 1991; Prasher, 1995). These tags can be added at the C- or N-terminus. For many purposes, C-terminal tagging is advantageous because the possibility for the tag to affect normal folding of polypeptide chain during translation is minimized and only full length protein could be detected. There are different modules available that can be used for the identification (Craven et al., 1998; Noguchi et al., 2008) and purification of protein and protein complexes in yeast (Tasto et al., 2001).

For the purpose of biochemical studies like immunoprecipitation, it is often desirable to have several fusion proteins tagged with various kinds of epitopes. Yeast cells efficiently carry out homologous recombination between short terminal homology regions on a linear PCR-derived DNA fragment and sequence on the chromosome (Baudin et al., 1993; Wach et al., 1997).

This approach allows manipulations of chromosomal genes, such as deletion, overexpression and tagging of gene products, without involving any cloning steps. There are many high-specificity monoclonal antibodies that are commercially available (Brizzard, 2008; Waugh, 2005) which can be very useful for the detection of the tag protein provided, if convenient template plasmid for PCR-based gene tagging is available.

To overcome this we have constructed plasmid containing FLAG tag module using two complementary primers, cloned into a plasmid containing kanMX6 module, which allows selection of G418-resistant cells in yeast. We have successfully FLAG tag *wat1/pop3* gene at its chromosomal locus and confirmed by western blot analysis. This construct can be very useful for the C terminal tagging of desired gene at its normal chromosomal locus.

MATERIALS AND METHODS

Strains and media

Standard bacterial culture media and growth conditions were used

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as described by Ausubel (1987). Standard culture media and methods were used for manipulation of yeast cells as described by Moreno et al. (1991).

Plasmid construction

DNA manipulation for cloning of DNA fragments were done as described by Sambrook et al. (1989). DNA fragments separated in agarose gels were purified by QIAquick gel extraction column (Qiagen). Oligonucleotides were synthesized by Sigma Aldrich. Long oligos were PAGE purified.

For construction of tag, two complementary primers coding for three tandem repeats of FLAG tag were mixed in distilled water. The mixture was heated at 90°C for 5 min and allowed to cool to 25°C. The resultant double-stranded DNA fragment was digested with PacI and AscI restriction enzyme, mixed with PacI and AscI digested vector, pFA6a 3HA-kanMX6 (Bahler et al., 1998) and ligated with T4 DNA ligase (New England Biolabs). The sequence of the inserted modules was confirmed by digesting with BglII and BamHI, since flag tag has only one BamHI site, as compared to HA tag construct which has two BamHI sites.

PCR amplification of DNA module for transformation

DNA fragment was amplified using Sprint Advantage Single Short PCR reaction mix (Clontech Laboratories) as described by manufacturer using pFA6a 3FLAG–kanMX6 plasmid as template. PCR conditions were as follows: a 94°C for 5 min denaturation step was followed by 35 cycles of 1 min at 94°C, 1 min at 55°C, 2 min at 68°C and a final extension of 5 min at 68°C. The products from three PCR reactions (2 µg of DNA) were pooled, precipitated with ethanol and dissolved in 10 µl of TE (pH 8) buffer (Sambrook et al., 1989). This concentrated DNA was used for transformation of *S. pombe* cells.

Transformation of *S. pombe* and selection of G418-resistant transformants

S. pombe cells were transformed with the PCR fragments as described by Bahler et al. (1998). In short, cells were washed once with 0.5 ml of LiAc/TE (100 mM Lithium acetate 1 mM TE) . The cell pellet was then resuspended in LiAc/TE at 2x109 cells/ml. 100 µl of the concentrated cells were mixed with 2 µl of sheared salmon sperm DNA (10 mg/ml- Sigma Aldrich) and 10 µl of the transforming DNA (1 to 2 µg).

After 10 min incubation at room temperature, 260 µl of 40% PEG/LiAc/TE was added. The cell suspension was mixed gently and incubated at 30°C for 1 h. 43 µl of DMSO was added, cells were heat shocked for 5 min at 42°C. Cells were then washed with 0.5 ml of water, resuspended in 100 µl of water and plated on two YEA plates. Plates were incubated at 30°C for 24 h, then replica plated on YEA plates containing 100 mg/l G418 (Sigma Aldrich). Plates were incubated for 2 to 3 days at 30°C and large colonies were streaked onto fresh YEA plates containing G418.

Validation of transformants for homologous integration by PCR

Genomic DNA of G418 resistant transformants was prepared as described by Moreno et al. (1991). Integration of the DNA fragment by homologous recombination was confirmed by PCR using Taq DNA polymerase with 3 µl genomic DNA (~20 ng), forward primer 5'-TCCAAATCAAGGTGAACTGC- 3' (present in coding region) and reverse primer 5'-CGAGGCAAGCTAAACAGATC- 3' (present in kanMX6 module) in a 25 µl reaction volume. PCR conditions were as follows: 25 cycles of 94°C for 1 min, 55°C for 1 min and 72°C for 2 min followed by an extension 72°C for 5 min.

Preparation of lysates and western blot analysis

Cells were harvested by centrifugation and lysed using glass beads and a vortex machine. Lysate in Phosphate Buffered Saline (PBS) was centrifuged at 10000 rpm in a microfuge for 5 min at 4°C. Supernatant was collected and protein estimation was performed using the Bradford assay method. For western blot analysis about 200 µg of total cell lysate was run on 10% SDS-PAGE, transferred to nitrocellulose and probed with anti Flag antibody (Sigma Aldrich cat. no. F1804). Horseradish peroxidase (HRP)-conjugated anti-mouse antibody (from Santa Cruz) was used as secondary antibodies. ECL Western blotting detection reagents (GE Healthcare) were used for protein detection using Kodak Biomax XAR film.

RESULTS AND DISCUSSION

Construction of PCR template plasmids for C-terminal epitope tagging

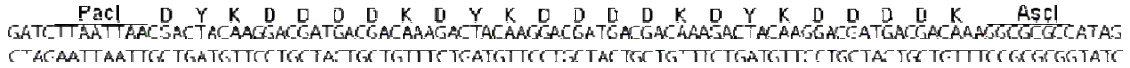
For simple and efficient biochemical analysis such as co-immunoprecipitation, protein complex purification and immunoflouresence studies, it is desirable to have several differentially tagged proteins in the same cells. Shorter tags such as HA, myc, flag generally perturb target protein function less, as compared to longer ones such as GFP, GST or MBP (Andresen et al., 2004). Many high specific antibodies and binding reagents for short defined peptide sequences are commercially available but only few of these antibodies are being used, due to non-availability of convenient plasmids template for PCR based tagging.

In order to add flag tag module in the existing list of module (Bahler et al., 1998), we annealed two complementary primers coding for three tandem repeats of Flag tag, digested with PacI and AscI restriction enzyme and cloned in pFA6a 3HA-kanMX6 plasmid, by digestion with PacI an AscI, thus replacing HA tag sequences with Flag tag sequences. Cloned plasmids were confirmed by digesting with BglII and BamHI restriction enzymes, since flag tag has only one BamHI site as compared to HA tag construct, which has two BamHI sites (data not shown). The tag sequence and resulting plasmids share the structure shown in Figure 1.

Validation of new tagging vector

To further confirm the utility of this plasmid, we successfully made flag tag at C terminus of our target gene *wat1/pop3* in fission yeast *S. pombe*. For this we amplified the FLAG tag module, using primer that creates 80 base pair of flanking sequences of target gene (wat1/ pop3) on both sides of the stop codon Table 1. Amplified

a



b

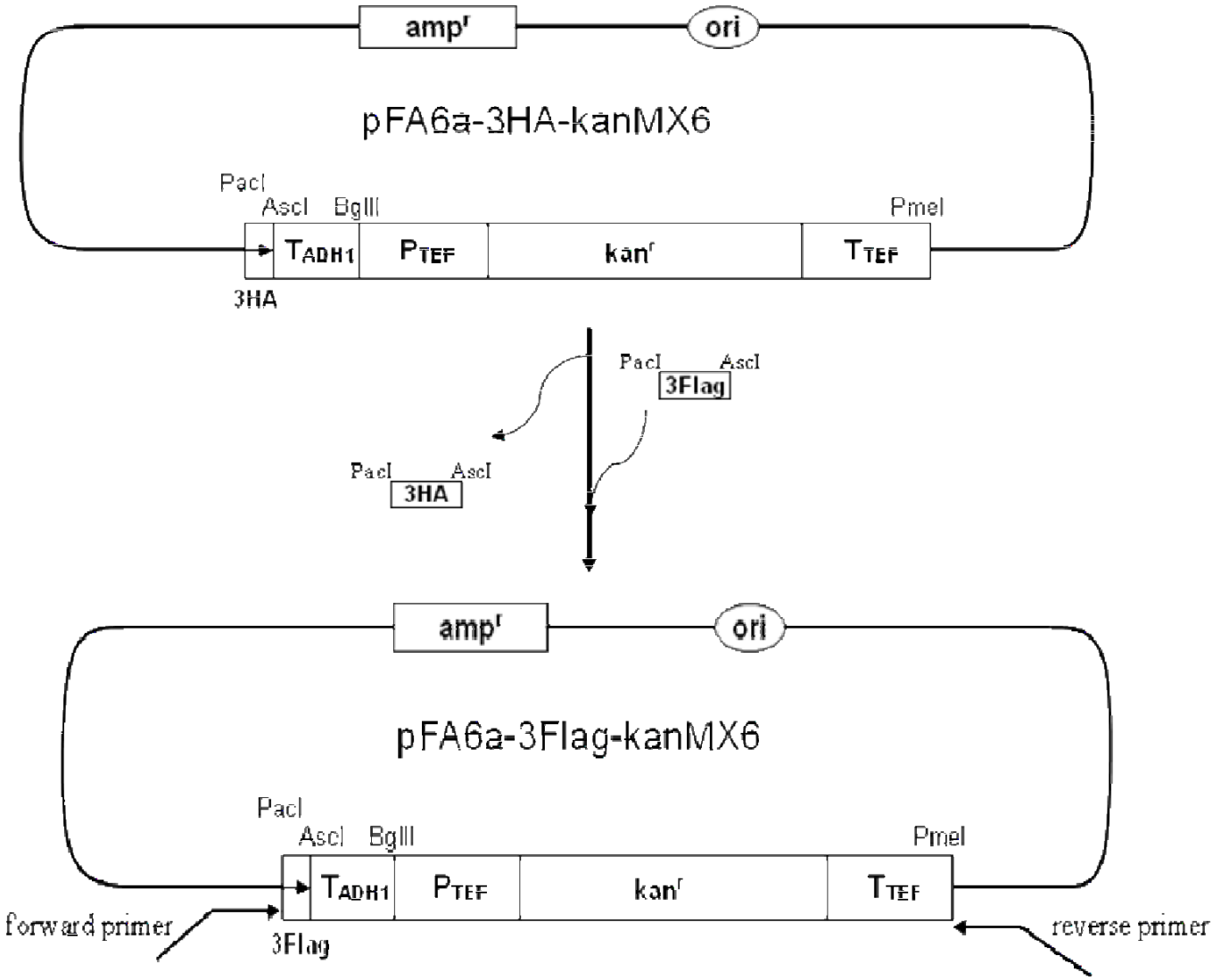


Figure 1a. Sequence of two primers showing three copies of Flag tag and restriction enzyme site used for cloning (b) HA tag module containing plasmid used for cloning (upper schematic) as described in materials and methods and plasmid containing Flag tag module (lower schematic). The position of forward and reverse primer for the amplification of module with overhang is shown.

Table 1. Primer used to amplify the module.

Forward primer	5'-(gene specific sequences)-CGG ATC CCC GGG TTA ATT AA-3'
Reverse Primer	5'-(gene specific sequences)-GAATTCGAGCTCGTTTAAAC-3'

PCR fragment was transformed in wild type *S. pombe* strain and G418 resistant transformants generated by homologous recombination were selected. The transformants were checked by PCR, using a primer set

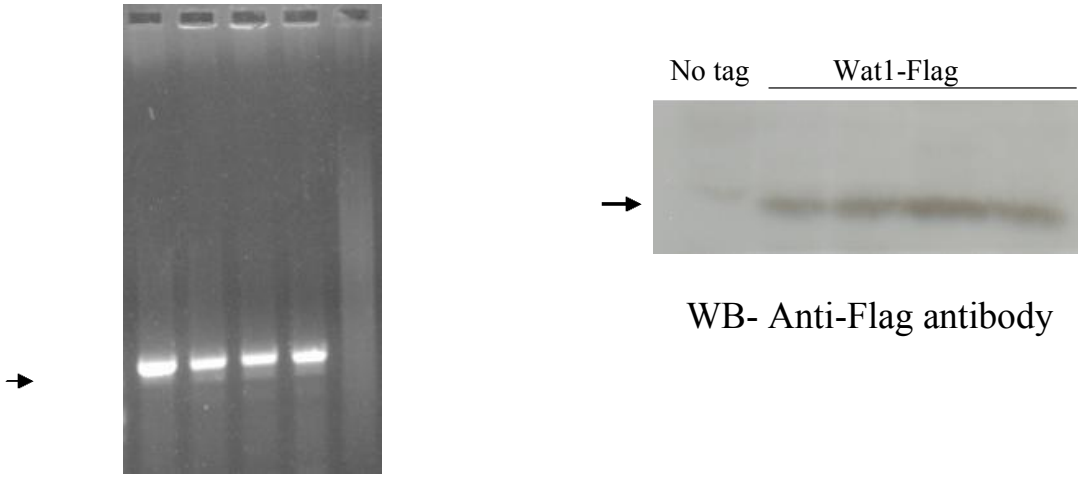


Figure 2. (a) Confirmation of integrants containing Flag tag: PCR was performed using genomic DNA as a template to ascertain the integration of flag tag module as described in materials and methods. PCR products were run on 1% agarose gel. PCR from the strain having flag tag (lane 1-4) and no tag (lane 5) (b) Western blot analysis of Flag tag *wat1/pop3* (lane 2-5) using anti flag antibody. A strain without any tag has been used as control (lane 1). Asterik (*) shows a cross reacting band.

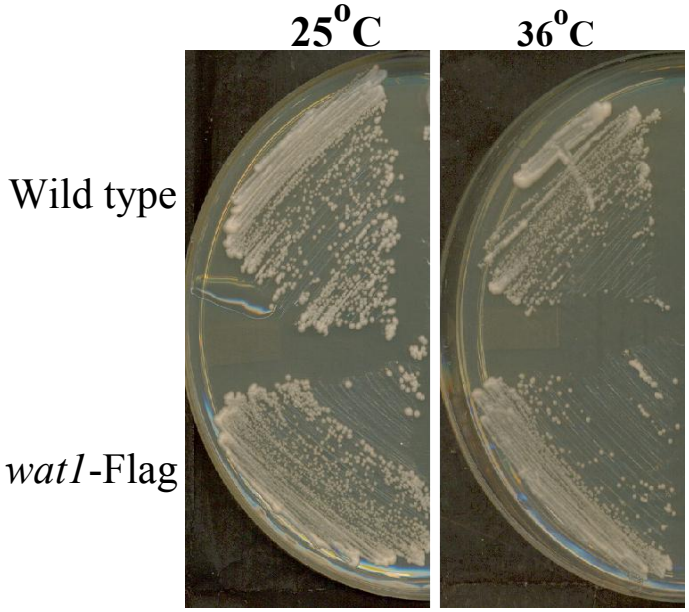


Figure 3. Addition of flag tag does not affect the function of the protein: Indicated strains were streaked on rich YEA plate and incubated at indicated temperature for 3-5 days before taking photograph.

that can amplify only the *wat1/pop3* integrants as 910 base pair band, as described in materials and methods (Figure 2A). Further western blot analysis was performed to check the expression of FLAG tag, using anti-FLAG antibody (Sigma, cat. no. F1804). As shown in Figure 2B, all the selected integrants were having FLAG tag as a 37 kd band (lane 2 to 6) which was absent in the strain having no tag (lane 1). Wat1-flag tag strain, grew at the same rate as the wild-type (WT) strain at different temperature (Figure 3), suggesting that addition of flag tag does not perturb the function of the protein.

This module can be very useful for making C -terminal tag with FLAG in any system where cells can be selected for G418 resistance. The efficiency of this method depends on the quality of long primer, PAGE purified primers that we have used, worked very well in most of the cases. Shorter primers can also be used for this method but the efficiency of homologous integration can vary with different cases. Though the epitope-tagging strategy can generate altered protein that can hamper its *in vivo* function but this can be readily resolved in yeast, using different genetic approaches. Another flag tag module has also been generated that contain five tandem repeats of flag epitope (Noguchi et al., 2008) while the module presented here contains three tandem repeats of flag epitope and that is sufficient to give enough signal for the detection.

Recently, a different FLAG tag module has been generated which also contain oligoglycine linker just before the tag (Funakoshi and Hochstrasser, 2009). Though oligoglycine linker can unfold the C- terminus of the protein for better accessibility of the tag but it may also hamper the structure of the protein, particularly at the C-terminus. Keeping this in mind, our Flag tag module is a simpler one that allows generating C terminal tagging of target gene at its normal chromosomal locus without interfering with its function. This plasmid can also be used with a recently described system for C-terminal epitope switching (Sung et al., 2008).

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