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Yeast Functional Analysis Report

HB tag modules for PCR-based gene tagging and tandem affinity purification in *Saccharomyces cerevisiae*

Christian Tagwerker[1,2], Hongwei Zhang[1], Xiaorong Wang[4], Liza S. Z. Larsen[5], Richard H. Lathrop[3], G. Wesley Hatfield[5], Bernhard Auer[2], Lan Huang[4] and Peter Kaiser[1]*

[1] *University of California Irvine, Department of Biological Chemistry, School of Medicine, 240D Med Sci I, Irvine, CA 92697-1700, USA*
[2] *Institute of Biochemistry, University of Innsbruck, Peter-Mayr-Strasse 1a, A-6020 Innsbruck, Austria*
[3] *University of California Irvine, Department of Computer Science, School of Information and Computer Sciences, Irvine, CA 92697-1700, USA*
[4] *University of California Irvine, Department of Physiology and Biophysics and Department of Developmental and Cell Biology, D224 Medical Science I, Irvine, CA 92697-1700, USA*
[5] *University of California Irvine, Department of Microbiology and Genetics, D224 Medical Science I, Irvine, CA 92697-1700, USA*

**Correspondence to:*
Peter Kaiser, University of California Irvine, Department of Biological Chemistry, School of Medicine, 240D Med Sci I, Irvine, CA 92697-1700, USA.
E-mail: pkaiser@uci.edu

Abstract

We have recently developed the HB tag as a useful tool for tandem-affinity purification under native as well as fully denaturing conditions. The HB tag and its derivatives consist of a hexahistidine tag and a bacterially-derived *in vivo* biotinylation signal peptide, which support sequential purification by Ni^{2+}-chelate chromatography and binding to immobilized streptavidin. To facilitate tagging of budding yeast proteins with HB tags, we have created a series of plasmids with various selectable markers. These plasmids allow single-step PCR-based tagging and expression under control of the endogenous promoters or the inducible *GAL1* promoter. HB tagging of several budding yeast ORFs demonstrated efficient biotinylation of the HB tag *in vivo* by endogenous yeast biotin ligases. No adverse effects of the HB tag on protein function were observed. The HB tagging plasmids presented here are related to previously reported epitope-tagging plasmids, allowing PCR-based tagging with the same locus-specific primer sets that are used for other widely used epitope-tagging strategies. The Sequences for the described plasmids were submitted to GenBank under Accession Numbers

DQ407918–pFA6a-HBH-kanMX6
DQ407919–pFA6a-HBH-hphMX4
DQ407920–pFA6a-HBH-TRP1

DQ407921–pFA6a-HTB-kanMX6
DQ407922–pFA6a-HTB-hphMX4
DQ407923–pFA6a-HTB-TRP1

DQ407924–pFA6a-BIO-kanMX6
DQ407925–pFA6a-BIO-hphMX4
DQ407926–pFA6a-BIO-TRP1

DQ407927–pFA6a-RGS18H-kanMX6
DQ407928–pFA6a-RGS18H-hphMX4
DQ407929–pFA6a-RGS18H-TRP1

DQ407930–pFA6a-kanMX6-PGAL1-HBH
DQ407931–pFA6a-TRP1-PGAL1-HBH

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Keywords: HB tag; epitope tagging; tandem affinity purification; PCR-mediated gene modification; biotin; HTB tag; HBH tag; HIS tag; BIO tag

Introduction

One-step PCR mediated tagging of open reading frames in yeast is a fast and efficient method to create valuable tools for protein analyses (Bahler *et al*., 1998; Knop *et al*., 1999; Longtine *et al*., 1998). The HB tag (HisBio tag) is a novel tandem affinity tag comprised of the hexahistidine tag and a 75 amino acid biotin tag derived from the *Propionibacterium shermanii* 1.3S transcarboxylase subunit (Cronan, 1990). The biotin tag serves as a recognition signal for endogenous biotin ligases that link biotin (vitamin H) through its carboxyl group to the lysine residue in position 41 in the biotin tag. Biotin ligases are found in both prokaryotic and eukaryotic cells and HB tag fusion proteins are efficiently recognized by biotin ligases *in vivo* in *Saccharomyces cerevisiae* and mammalian cells. HB-tagged proteins can be purified in a two-step purification procedure on Ni^{2+} chelate and streptavidin resins. Importantly, both resins tolerate fully denaturing conditions, such as 8 M urea or 6 M guanidinium chloride, wash steps involving various detergents, high salt concentrations and organic solvents. These stringent conditions guarantee preservation of posttranslational protein modifications and a high level of purity, which facilitates mass spectrometric analyses (Tagwerker *et al*., 2006). The HB tag has also proved to be a valuable tool in combination with cross-linking reagents to study non-covalent, transient protein–protein interactions (Guerrero *et al*., 2005). Therefore, the HB tag extends application of tandem-affinity purification strategies to experimental approaches that benefit from highly denaturing conditions (Tagwerker *et al*., 2006; Guerrero *et al*., 2005). Although the HB tag can in principle also be used for purification of protein complexes under native conditions, we cannot see any obvious advantage over the widely used TAP tag (Puig *et al*., 2001) for native purifications. However, we have not directly compared the HB tag and TAP tag under these conditions.

Here we describe a series of plasmids for HB tagging by one-step PCR-mediated modification of chromosomal genes in *S. cerevisiae*. We constructed several useful derivatives of the HB tag, including the HBH tag, which consists of the BIO tag flanked by two hexahistidine tags. The additional hexahistidine tag significantly improves purification by Ni^{2+}-chelate chromatography. The HTB tag includes a tobacco etch virus protease cleavage site (TEV site) to allow protease-driven elution of the tagged proteins from streptavidin resins. The plasmids contain one of three selectable markers, the *kanMX6* module (Wach *et al*., 1994), the *hphMX4* module (Goldstein and McCusker, 1999) and the *S. cerevisiae TRP1* gene (Longtine *et al*., 1998) for selection with G418, hygromycine, or growth media lacking the amino acid tryptophan, respectively. The plasmids are useful for carboxyl-terminal tagging as well as amino-terminal tagging combined with regulated expression under control of the *GAL1* promoter.

Materials and methods

Growth media and yeast strains

All yeast strains used in this study were isogenic to 15DaubΔ, bar1Δ ura3Δns, a derivative of BF264-15D (Table 2) (Reed *et al*., 1985). Strains were grown in standard culture media (Guthrie and Fink, 1991).

HTB-sequence assembly

The full-length 365 base pair RGS–6HIS–TEV–BIO (HTB) was assembled by the ligation of five abutting intermediate DNA fragments. Each intermediate DNA fragment was produced by primer extension of four to five CODA (computationally optimized DNA assembly)-designed, overlapping and abutting self-assembling oligonucleotides (Table 1) according to the methods described by Larsen *et al*. (manuscript in preparation). The oligonucleotide set for each intermediate DNA fragment (Table 1) was added at a final concentration of 0.1 μM to a 50 μl primer extension and PCR amplification reaction solution along with an excess (1 μM) of leader and trailer primer oligonucleotides (the 5′-most and the 3′-most distant oligonucleotides). Each self-assembling oligonucleotide set was extended into an intermediate DNA fragment in a reaction containing: 0.7 U DyNAzyme EXT polymerase (Finnzymes, Espoo, Finland), 1.5 mM $MgCl_2$ and 300 μM dNTPs under the following reaction conditions: 5 min 94 °C denaturation, followed by 30 cycles of 94 °C for 1 min, 45 °C for 0.5 min, 72 °C for 0.5 min. The reactions were concluded with a 10 min extension step at 72 °C.

The five resulting intermediate DNA fragments were used to construct the PacI–HTB–AscI fragment by ligating fragment 0 to fragment 1,

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Table 1. Oligonucleotides used to assemble intermediate DNA fragments (0–4) in a series of phosphorylation, ligation and PCR reactions for PacI–RGS6H–TEV–BIO (HTB)-AscI fragment construction

Intermediate DNA fragment	Oligo	Orientation	Length	Sequence (5′ to 3′)
0	0-0	Antisense	19	GATGTGAACCCCTGTTAAT
	0-1	Sense	32	TAACAGGGGTTCACATCATCACCACCATCATG
	0-2	Antisense	31	GTGGGTATATCGTAGTCATGATGGTGGTGAT
	0-3	Sense	31	ACTACGATATACCCACAACCGCCAGCGAGAA
	0-4	Antisense	35	ACTCTGATTGAAAGTACAGATTCTCGCTGGCGGTT
1	1-0	Sense	35	TCTGTACTTTCAATCAGAGTTAAAAACCGCCGCAC
	1-1	Antisense	32	CTCGTCATGTTGAGCTAGTGCGGCGGTTTTTA
	1-2	Sense	32	TAGCTCAACATGACGAGGCTGCTGGAAAGGCC
	1-3	Antisense	35	GCAGGGATTTCACCTTCACCGGCCTTTCCAGCAGC
2	2-0	Sense	35	GGTGAAGGTGAAATCCCTGCCCCTCTTGCTGGTAC
	2-1	Antisense	32	CCAGTATCTTAGAAACGGTACCAGCAAGAGGG
	2-2	Sense	32	CGTTTCTAAGATACTGGTAAAAGAAGGTGACA
	2-3	Antisense	35	TGTTTGACCAGCTTTAACAGTGTCACCTTCTTTTA
3	3-0	Sense	35	CTGTTAAAGCTGGTCAAACAGTTCTGGTGCTGGAG
	3-1	Antisense	32	GTCTCCATTTTCATAGCCTCCAGCACCAGAAC
	3-2	Sense	32	GCTATGAAAATGGAGACAGAAATTAACGCTCC
	3-3	Antisense	35	TTTCAACTTTTCCGTCAGTAGGAGCGTTAATTTCT
4	4-0	Sense	35	TACTGACGGAAAAGTTGAAAAGGTGTTAGTTAAGG
	4-1	Antisense	32	TTGAACAGCATCTCTTTCCTTAACTAACACCT
	4-2	Sense	32	AAAGAGATGCTGTTCAAGGTGGTCAAGGTCTA
	4-3	Antisense	42	CGCGCCTCATTAAACGCCGATCTTGATTAGACCTTGACCACC
	4-4	Sense	23	ATCAAGATCGGCGTTTAATGAGG
PacI–RGS6H primer	469	—	27	TTAATTAACAGGGGTTCACATCATCAC
BIO–AscI primer	470	—	27	GGCGCGCCTCATTAAACGCCGATCTTG
BIO–6H–AscI primer	471	—	45	GGCGCGCCTCATTAATGATGGTGGTGATGATGAACGCCGATCTTG

and fragment 2 to fragment 3 (see Table 1). The products of each ligation reaction were isolated by agarose gel electrophoresis. The ligation product of the intermediate DNA fragments 0 and 1 and the ligation product of the intermediate DNA fragments 2 and 3 were isolated by agarose gel electrophoresis and ligated to one another. This ligation product was isolated by agarose gel electrophoresis and PCR-amplified using 1 μM oligos 0-0 and 3-3 (Table 1), each as primers in a 20 μl PCR reaction as described above. Finally, the ligation product of intermediate DNA fragments 0, 1, 2 and 3 were ligated to intermediate DNA fragment 4. This final 365 bp product was isolated by agarose gel electrophoresis and PCR-amplified in a 50 μl PCR reaction at an annealing temperature of 55 °C for 1 min using 1 μM each of primers 469 and 470 (Table 1), as described above. This final PacI–HTB–AscI PCR product was isolated by agarose gel electrophoresis and ligated into a bacterial vector for sequence confirmation.

The 296 bp PacI–HBH–AscI sequence was obtained by amplifying RGS6H–biotin DNA from a bacterial vector using primers 469 and 471 (Table 1).

Plasmid constructions

Plasmids pFA6a–kanMX4, pFA6a–kanMX6 (Wach *et al*., 1994, 1997), pFA6a–hphMX4 (Goldstein and McCusker, 1999) and pFA6a–13Myc–TRP1 (Longtine *et al*., 1998) have been described previously.

Plasmids pFA6a–HBH–kanMX6 and pFA6a–HTB–kanMX6 were constructed by ligating PacI–HBH/HTB–AscI fragments into PacI–AscI-digested pFA6a–kanMX6. These plasmids were then used to switch the selectable marker following the HBH or HTB tags. pFA6a–HBH/HTB–TRP1 were constructed by ligating a BglII–PmeI fragment containing the *TRP1* module of pFA6a -13Myc–TRP1 with BglII–PmeI-digested pFA6a–HBH/HTB–kanMX6 plasmids. Similarly, pFA6a–HBH/HTB–hphMX4 were constructed by inserting BglII–PmeI fragments derived from pFA6a–hphMX4 into BglII–PmeI-digested pFA6a–HBH/HTB–kanMX6 plasmids.

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Pac1–RGS18HIS–AscI and PacI–BIO–AscI fragments were each cloned into PacI–AscI-digested pFA6a–kanMX6/hphMX4/TRP1 vectors to generate a series of plasmids with different tags.

To generate plasmids for amino-terminal tagging and expression under *GAL1* control, pFA6a–kanMX6–PGAL1–3HA (Longtine *et al*., 1998) was digested with PacI and AscI to exchange the HA tag with a PacI–HBH–AscI fragment. This resulted in generation of pFA6–kanMX6–PGAL1–HBH. pFA6–TRP1–PGAL1–HBH was created by replacing the PacI–3HA–AscI fragment with PacI–HBH–AscI in pFA6a–TRP1–PGAL1–3HA (Figure 1).

HBH and HTB PCR-mediated gene modification

The 20 nucleotides underlined in the primer sequences in Table 2 indicate sequence homology to the HB tag/marker cassette. Primers used for C-terminal and N-terminal tagging modules are compatible with the primers described by Longtine *et al*. (1998). C-terminal tagging primers are compatible with primers F2 and R1 as described in Longtine *et al*. (1998). The 5′ N-terminal tagging primers correspond to primer F4 in Longtine *et al*. (1998). The reverse primer for N-terminal tagging is specific for the tag used. The remaining 40 nucleotides of the primers are locus-specific sequences (non-underlined sequences in Table 2).

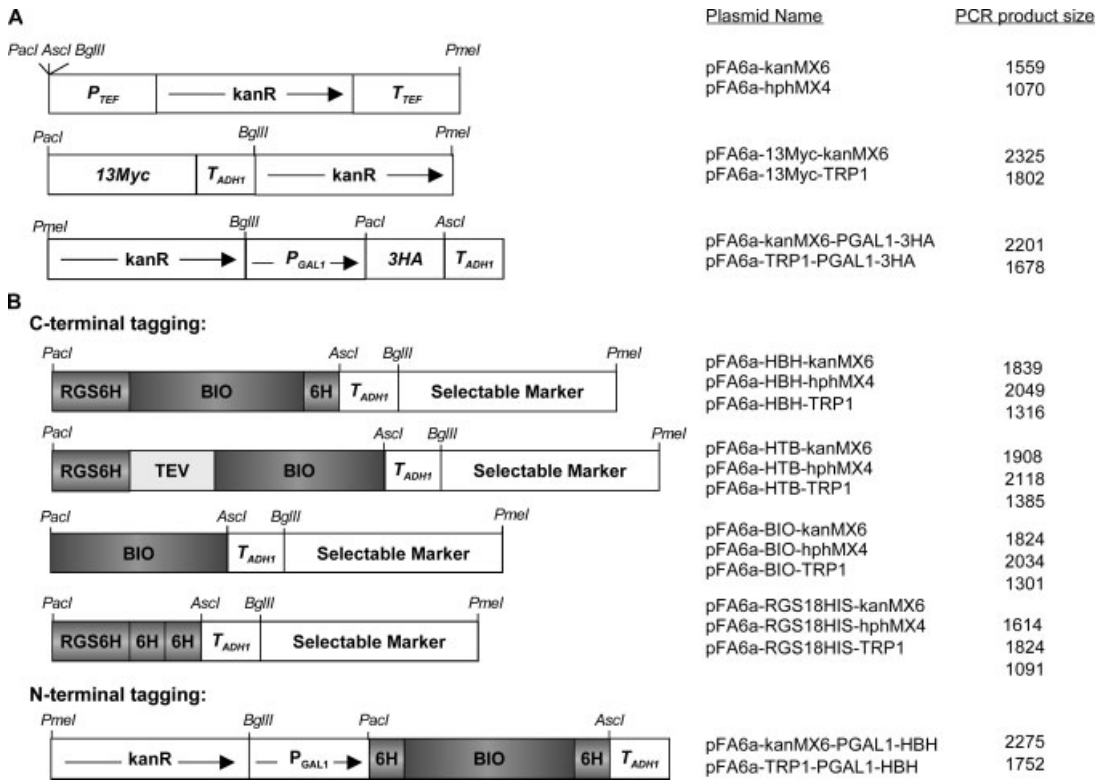

Figure 1. HB-module templates for PCR-mediated modification in *Saccharomyces cerevisiae*; PCR product sizes (bp) are calculated assuming that each primer includes 40 nucleotides of homology to the target sequence. Restriction sites used for cloning are indicated. Arrows within the boxes indicate the direction of transcription. (A) PCR template modules provided by Longtine *et al.* (1998) and Goldstein and McCusker (1999), from which all templates described in this study were derived. (B) Various derivatives of the HB tag for expression of amino-terminal (pFA6a–kanMX6–PGAL1–HBH and pFA6a–TRP1–PGAL1–HBH) or carboxyl-terminal tagged proteins. HBH and HTB tagging allows tandem-affinity purification of the tagged proteins by Ni^{2+} chelate chromatography and binding to streptavidin resins. HTB-tagged proteins can be eluted from streptavidin beads by protease cleavage with TEV protease. BIO and RGS18H tagging constructs can be useful for the 'split-tag' approach (Puig *et al*., 2001) to selectively isolate protein complexes containing two given proteins. For further explanation, see Results and discussion section

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Table 2. Primers used for N-terminal and C-terminal tagging of Met30, Cdc4, Pre1 and Rpn11

Tagging	ORF/ Primer		Sequence
N-terminal			
	MET30		
	525	Forward	5′-CACGTGATCGGGAAGCCACAGTTTGCGCGGAGATATTTTAGAATTCGAGCTCGTTTAAAC-3′
	624	Reverse	5′-CCTTGTCCTCGAAACTCATCATCCTTTGCCTCTCTCTCCTGGCTCCATGATGGTGGTGAT-3′
	CDC4		
	527	Forward	5′-CAATCCTATGGAGGAAGGAGCGCTGCCGTTATCACCCACTGAATTCGAGCTCGTTTAAAC-3′
	623	Reverse	5′-GATATCACGTAATGGAAACTCAGCTAAGGGAAACGACCCGGCTCCATGATGGTGGTGAT-3′
C-terminal			
	PRE1		
	655	Forward	5′-TAAAGATGGCATAAGACAAGTAGATGACTTCCAGGCACAGCGGATCCCCGGGTTAATTAA-3′
	656	Reverse	5′-GCAATCACCTTTTCCGTGTGATTACACTGAATATCTTTCAGAATTCGAGCTCGTTTAAAC-3′
	RPN11		
	538	Forward	5′-TTCTGTGCTGACGGCGGGTGTTAATTCAGTGGCAATTAAACGGATCCCCGGGTTAATTAA-3′
	539	Reverse	5′-TTTCTAGTTATTTAATGCATAATGACTTTATAAAATTTGTGAATTCGAGCTCGTTTAAAC-3′

The 20 nucleotide sequences annealing to the plasmid templates are underlined.

DNA for PCR-mediated modification of all subsequent ORFs was amplified in 300 µl reactions containing ~0.1 µg plasmid DNA, 1 µM each primer, 2.5 mM $MgCl_2$, 0.2 mM each dNTP, 30 µl 10 × Ex Taq buffer (Takara Bio Inc., Shiga, Japan). PCR was started by addition of 7.5 U Takara Ex Taq polymerase in the following reaction conditions: 5 min 94 °C denaturation, followed by five cycles of 94 °C for 1 min, 45 °C for 1 min, 72 °C for 2 min and 24 amplification cycles at 94 °C for 1 min, 60 °C for 1 min, 72 °C for 2 min. Reactions were concluded with a 10 min 72 °C extension step. PCR products were ethanol-precipitated with 0.3 M NaOAc and the pellet solubilized in 20 µl TE (10 mM Tris–HCl, 1 mM EDTA, pH 8). 5–10 µl precipitated DNA was used per yeast transformation, as described by Hill *et al*. (1991).

HBH-tagging and promoter replacement with *GAL1* of *MET30* and *CDC4* (strains PY1267 and PY1276; see Table 3) was carried out by amplifying DNA from pFA6–TRP1–PGAL1–HBH, using primer pairs 525/624 and 527/623, respectively. Amplified DNA was transformed into yeast and transformants were selected on galactose plates lacking tryptophan to induce expression of the essential genes *MET30* and *CDC4*.

Rpn11 and Pre1 were tagged C-terminally with HBH and HTB, respectively, after transformation of DNA amplified from pFA6a–HBH and pFA6a–HTB plasmids, respectively (primer pairs 538/539 and 655/656; see Table 2).

Table 3. Yeast strains used in this study

Strain	Relevant genotype	Source
15Daub	*a bar1* Δ *ura3*Δ*ns ade1 his2 leu2-3,112 trp1-1a*	(Reed *et al*., 1985)
PY236	*a bar1 pep4::URA3*	(Kaiser *et al*., 1999)
PY1245	*a bar1 pep4::URA3 RPN11-HBH::TRP1*	This study
PY1246	*a bar1 pep4::URA3 RPN11-HBH::HYG*	This study
PY1247	*a bar1 pep4::URA3 RPN11-HBH::KAN*	This study
PY1248	*a bar1 pep4::URA3 PRE1-HTB::TRP1*	This study
PY1249	*a bar1 pep4::URA3 PRE1-HTB::HYG*	This study
PY1250	*a bar1 pep4::URA3 PRE1-HTB::KAN*	This study
PY1276	*a bar1 pep4::URA3 GAL1-HBH-CDC4::TRP1*	This study
PY1267	*a bar1 pep4::URA3 GAL1-HBH-MET30::TRP1*	This study
PY1500	*a bar1 pep4::URA3 RPN11-RGS18H::KAN*	This study

HTB, RGS6H–TEV–biotin; HBH, RGS6H–biotin–6 × H.

Ni^{2+} chelate chromatography of Rpn11-RGS18H

Cells expressing C-terminal RGS18H-tagged Rpn11 were grown in 50 ml YEPD to $A_{600} = 0.7$; the cells were harvested by filtration, washed in ice-cold water and frozen at −80 °C. The cells were lysed with glass beads for 160 s (setting 4.5) in a FastPrep FP120 (Qbiogene, Carlsbad, CA) in buffer A (8 M urea, 300 mM NaCl, 0.5% NP-40, 50 mM NaH_2PO_4) + 1 mM PMSF. Cell debris was removed by centrifugation for 10 min at 16 100 × *g*. The whole cell lysate was incubated with Ni^{2+}-Sepharose 6 FastFlow resin (GE Healthcare) overnight at room temperature (15 µl 50%

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slurry for each mg of total protein lysate). The resin was washed in column format sequentially with 20 bed volumes each of buffer A, pH 8, buffer A pH 6.3 containing 10 mM imidazole and buffer A, pH 5. PMSF was omitted in wash buffers. Elution was carried out with 20 bed volumes of buffer A, pH 4.3.

Streptavidin chromatography and TEV digestion of Pre1-HTB

Cells expressing C-terminal HTB-tagged Pre1 were lysed as described above in 600 µl 1 × TEV buffer (50 mM Tris, 0.5 mM EDTA, pH 8) at 4 °C. After removal of the glass beads the lysate was centrifuged for 10 min at 16 100 × *g* at 4 °C. 600 µl cleared lysate (equivalent to 2 mg total protein) was incubated with immobilized Streptavidin (100 µl slurry pre-equilibrated in 1 × TEV buffer) (Pierce, Rockford, IL) overnight at 4 °C. Streptavidin beads were washed in 6 × 0.5 ml TEV buffer. For the TEV cleavage reaction, 15 µl 20 × TEV Buffer, 3 µl 0.1 mM DTT and 5 µl AcTEV™ Protease (10 U/µl; Invitrogen, Carlsbad, CA) were added to 100 µl bead slurry. The end volume was adjusted to 300 µl with water and TEV digestion was carried out at 30 °C for 1 h. After gentle centrifugation of the bead solution (100 × *g*) a 50 µl aliquot was removed from the supernatant and digestion was continued overnight at 4 °C. Another 50 µl aliquot was taken and samples were separated by SDS–PAGE and analysed by immunoblotting as described below.

Protein analyses

For immunoblot analysis, protein extracts were prepared in urea-buffer (8 M urea, 300 mM NaCl, 50 mM NaH_2PO_4, 0.5% NP-40, 1 mM phenylmethylsulphonyl fluoride [PMSF], pH 8). Cells were broken with glass beads for 160 s at setting 4.5 in a FastPrep FP120 (Qbiogene, Carlsbad, CA) and cell debris was removed by centrifugation for 10 min at 16 100 × *g*. Protein lysates were diluted to a final concentration of 4 M urea before separation by SDS–PAGE. Separated proteins were transferred to a polyvinylidene difluoride membrane and the membrane was probed with the anti-RGS6H antibody (1 : 2000; Qiagen, Germantown, MD) to detect the RGS6H epitope, or a streptavidin–HRP conjugate was used (1 : 15 000, working concentration 0.6 µg/ml; Pierce, Rockford, IL) to detect biotinylated proteins.

Spotting experiments

Cells were grown overnight in liquid YPGal at 30 °C. Cultures were then diluted to a concentration of 5×10^7 cells/ml, and four 10-fold serial dilutions were made; 3 µl of each dilution were spotted onto YPD and YPGal agar plates and grown at 30 °C for 3 days.

Results and discussion

The HB tandem-affinity tag is a useful addition to the collection of existing epitope tags (Bahler *et al.*, 1998; Knop *et al.*, 1999; Longtine *et al.*, 1998). Particularly proteomic analyses of posttranslational modifications benefit from the stringent purification conditions that are compatible with the HB tag and its derivatives (Tagwerker *et al.*, 2006). We have used a subset of widely used plasmids with varying selectable markers and combined them with the HB tag for rapid PCR-based tagging and analysis of yeast proteins.

The HB tags combine hexahistidine tags with an *in vivo* biotinylation signal. Instead of a simple hexahistidine sequence, we used the RGS6H epitope that includes an additional three amino acids and forms a specific epitope that can be efficiently detected by a commercially available antibody. Sequences encoding HB tags were optimized for codon usage in yeast to eliminate possible effects on protein expression due to codon bias (Larsen *et al.*, manuscript in preparation). C-terminal tagging and purification using the HBH-tag (RGS6H–BIO–6 × HIS) has been described previously (Guerrero *et al.*, 2005; Tagwerker *et al.*, 2006). In addition, the use of N-terminally HBT-tagged (RGS6H–BIO–TEV) ubiquitin in proteome-wide ubiquitin profiling has been reported (Tagwerker *et al.*, 2006). Here we describe the generation of HBH-tagging plasmids with additional selection markers for C-terminal tagging. We also report sets of plasmids for N-terminal HBH-tagging, C-terminal HTB-tagging, C-terminal BIO-tagging and C-terminal RGS18HIS tagging (Figure 1B). The individual tags are described in more detail below.

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The HBH tag contains two hexahistidine sequences that flank the BIO tag. The additional hexahistidine sequence significantly increases purification efficiency by Ni^{2+}-chelate chromatography, particularly for purification under fully denaturing conditions (data not shown; and Qiagen, 2003). After elution of HBH-tagged proteins from Ni^{2+}-chelate resins, HBH-tagged proteins can be further purified by binding to streptavidin beads. The extremely high-affinity interaction between the biotin molecule attached to the HBH tag and the streptavidin resin allows exceptionally stringent wash conditions. However, because the biotin–streptavidin interaction is virtually irreversible, elution of HBH-tagged proteins from streptavidin beads is inefficient (Holmberg *et al*., 2005; Nikolau *et al*., 1985; Savage, 1994). If the purified proteins are used for mass spectrometric analyses, elution from streptavidin beads is not required because trypsin digestion can be performed efficiently on bead-bound proteins and the released peptides can be analysed by mass spectrometry (Guerrero *et al*., 2005; Kho *et al*., 2004; Rybak *et al*., 2005; Tagwerker *et al*., 2006). For applications where elution of biotinylated proteins is required, affinity resins using monomeric avidin instead of streptavidin can be used. An excess of biotin or SDS will efficiently elute biotinylated proteins from monomeric avidin resins (Swack *et al*., 1978). However, the monomeric avidin–biotin interaction is significantly weaker than the streptavidin–biotin interaction and cannot tolerate denaturing purification conditions (Green, 1990).

The HTB tag was constructed to maintain the benefits of the high-affinity streptavidin–biotin interaction and at the same time allow elution of C-terminally HTB-tagged proteins by site-specific proteolytic cleavage with TEV protease (Dougherty *et al*., 1988).

To test the generated plasmids, HBH- and HTB-containing plasmids were used as templates for one-step PCR-mediated tagging of *PRE1*, a 20S proteasome β-type subunit, and *RPN11*, a metalloprotease subunit of the 19S regulatory particle of the 26S proteasome. HTB plasmids with all three selectable markers were fused to Pre1 ($Pre1^{HTB}$) and HBH plasmids with all three selectable markers were fused to Rpn11 ($Rpn11^{HBH}$). Analysis of whole cell lysates from yeast strains expressing $Pre1^{HTB}$ and $Rpn11^{HBH}$ by immunoblotting confirmed tagging in each strain as well as efficient biotinylation (Figure 2). When whole

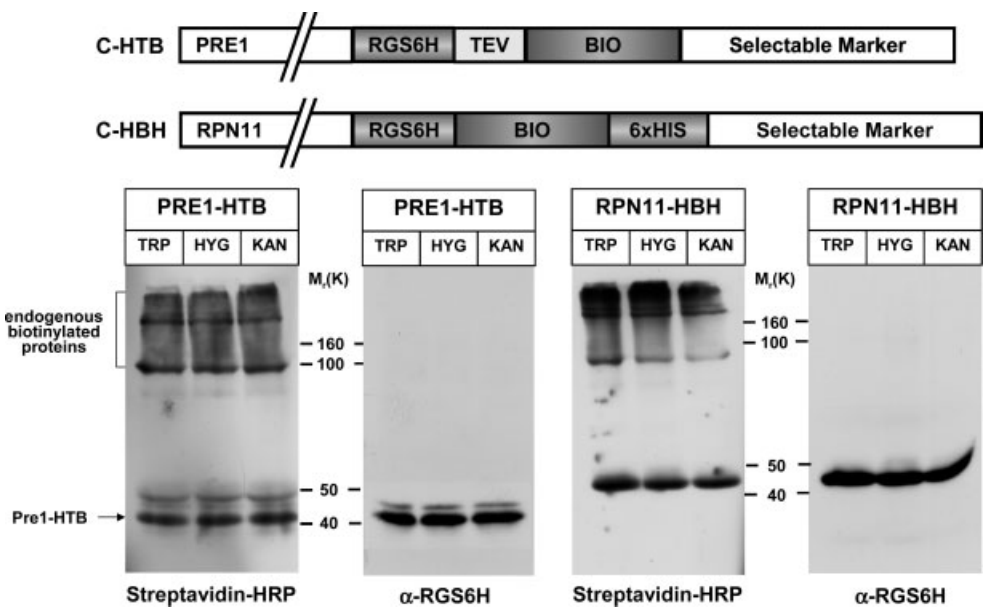

Figure 2. C-terminally HTB-tagged Pre1 and HBH-tagged Rpn11. Both open reading frames were modified using one-step PCR-based gene modification with DNA derived from six different plasmids, encoding the *S. cerevisiae TRP1* gene (TRP), the *kanMX6* (KAN) or *hphMX4* (HYG) modules trailing the HTB or HBH coding sequence. Immunoblotting with anti-RGS6H and streptavidin–HRP confirmed all plasmids expressing tagged Pre1 or Rpn11 and biotinylation of these constructs *in vivo*. Note that detection with streptavidin–HRP also detects endogenous biotinylated proteins

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cell lysates were analysed with streptavidin–HRP conjugates several endogenous biotinylated proteins can be detected. All eukaryotes express between four and six proteins that are biotinylated (Chandler and Ballard, 1988; Lim *et al.*, 1987; Nikolau *et al.*, 1985) (cf. anti-RGS6H and streptavidin–HRP detection in Figure 2). Purification of HBH-tagged proteins, including Rpn11 by Ni^{2+}-chelate chromatography and binding to streptavidin beads, has been reported in detail previously (Guerrero *et al.*, 2005; Tagwerker *et al.*, 2006).

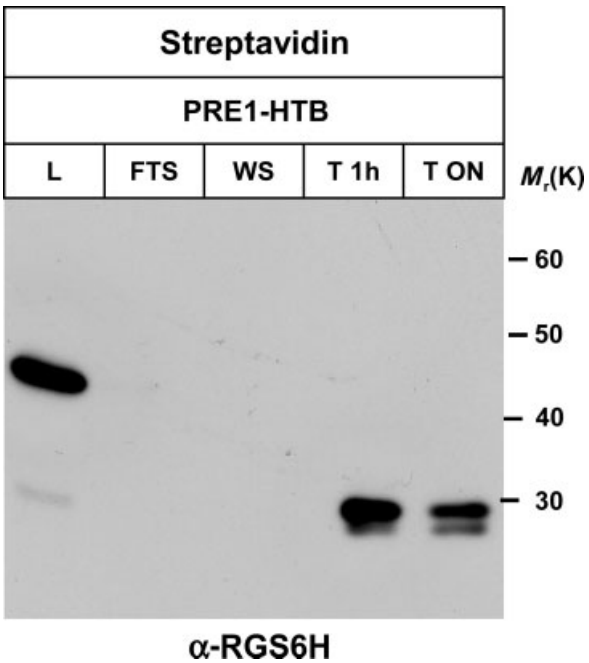

Figure 3. Streptavidin chromatography of HTB-tagged Pre1. Chromatography fractions were detected by immunoblotting with anti-RGS6H. C-terminally HTB-tagged Pre1 ($Pre1^{HTB}$) was bound to streptavidin resin in TEV buffer (L, load). Binding was efficient, as indicated by the absence of $Pre1^{HTB}$ from the flow-through (FTS). After extensive washing with 30 bed volumes (WS), digestion for 1 h at 30 °C with AcTEV™ protease (T = 1 h) was sufficient to recover over 90% 6 × His-tagged Pre1 (30 kDa protein species). Digestion overnight at 4 °C (T ON) did not increase yield significantly

To test elution of HTB-tagged proteins from streptavidin resins by site-specific proteolysis with TEV protease, $Pre1^{HTB}$ was purified from total cell lysates by binding to streptavidin beads, and streptavidin-bound $Pre1^{HTB}$ was incubated with TEV protease (Figure 3). Over 95% of the resulting RGS6H-tagged Pre1 was released from the streptavidin-bound $Pre1^{HTB}$ by TEV cleavage after 1 h digestion at 30 °C. Digestion for 12 h at 4 °C was equally efficient (Figure 3). Because the RGS6H tag is retained on the eluted proteins, further purification by Ni^{2+}-chelate chromatography is possible.

HB tags add around 8 kDa to tagged proteins. While the HB tags are considerably smaller than some other tags (GFP, GST), it was important to verify that HB tagging does not generally affect protein function or yeast cell physiology. HB tagging of essential proteins, such as 19S proteasome subunits Rpn11 and Rpt5, the 20S proteasome subunit Pre1, the F-box proteins Cdc4 and Met30 (Figure 4) and the ubiquitin-like protein Smt3 (yeast homologue of mammalian SUMO) have shown no obvious effects on cell growth. Several HB-tagged proteins (Skp1, Pre1, Rpn11, Rpt5 Smt3), were expressed under control of their native promoter and no obvious growth defects were observed, suggesting full functionality (data not shown). Furthermore, HB-tagged ubiquitin as

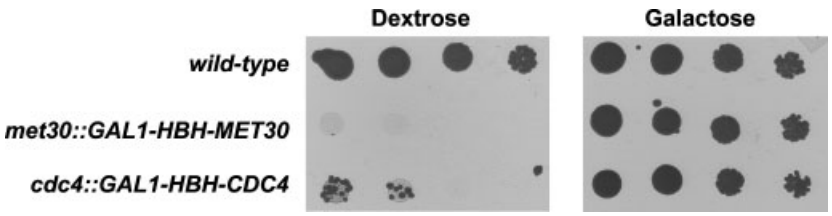

Figure 4. Growth analysis of cells expressing HBH-tagged Met30 and Cdc4. *MET30* and *CDC4* were modified with the *GAL1* promoter and HBH tag on their N-termini using one-step PCR-based gene modification with DNA derived from pFA6–TRP1–PGAL1–HBH. Primers were designed to replace the endogenous promoters of *MET30* and *CDC4*. Therefore, ^{HBH}Met30 or ^{HBH}Cdc4 was the only source of Met30 or Cdc4, respectively, in these cells. Met30 and Cdc4 are essential and HBH-tagged versions rescued the growth-deficient mutants when expression was induced on galactose-containing plates, indicating that the HBH tag did not affect function of these proteins. Ten-fold dilutions were spotted on the rich media plates containing dextrose or galactose as indicated

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well as HB-tagged Smt3 were conjugated to cellular proteins, further demonstrating that the HB tag has little effect on protein function (Tagwerker *et al*., 2006; and data not shown).

Importantly, we noticed that when cells expressing high levels of HB-tagged proteins were grown in synthetic minimal media, biotinylation efficiency of the HB tag was reduced *in vivo* (Tagwerker *et al*., 2006). We believe that this was a result of relatively low biotin concentrations in minimal growth media, because these effects could be completely reverted by supplementation of the growth media with 4 μM biotin (Tagwerker *et al*., 2006).

In addition to the HB-tagging modules described above, we also generated tagging plasmids where the two components of the HB tag are separated. These plasmids contain either a polyhistidine tag or the BIO tag (Figure 1). Separation of the tandem-affinity tag can be useful for split-tag approaches to identify protein complexes that contain two given proteins (Puig *et al*., 2001). One series of plasmids contains the BIO tag, for tagging and purification by binding to streptavidin resins (Figure 1). The other series of plasmids containing the polyhistidine tag for purification on Ni^{2+} chelate resins were constructed to contain the RGS6HIS epitope as part of a series of 18 histidine residues (RGS18HIS) (Figure 1). We noticed that proteins tagged with RGS18H bind tightly to Ni^{2+} chelate resins and allow very stringent wash conditions to significantly decrease the high background usually associated with purification of 6 × histidine-tagged proteins from yeast cell extracts. Proteins tagged with a 6 × histidine tag usually elute from Ni^{2+} chelate resins at pH < 6.2, whereas 12 × histidine tagged proteins (e.g. HBH-tagged) elute at pH < 5.8 (Qiagen, 2003; and data not shown). RGS18HIS-tagged proteins tolerated even more stringent wash conditions, as demonstrated for C-terminally RGS18HIS-tagged Rpn11 in Figure 5. Ni^{2+} chelate chromatography of $Rpn11^{RGS18H}$ was performed in 8 M urea buffers and a series of wash steps with decreasing pH demonstrated minimal loss of bound protein, even at pH as low as 5.0 (Figure 5). Elution of $Rpn11^{RGS18H}$ at pH 4.3 recovered close to 100% of the fusion protein.

In summary, we describe here a series of plasmids for one-step PCR-based tagging of yeast proteins with the HB tag and its derivatives. These plasmids are compatible with the widely used tagging plasmids developed by Longtine *et al*. (1998) and extend this collection to provide useful tools for the study of the yeast proteome.

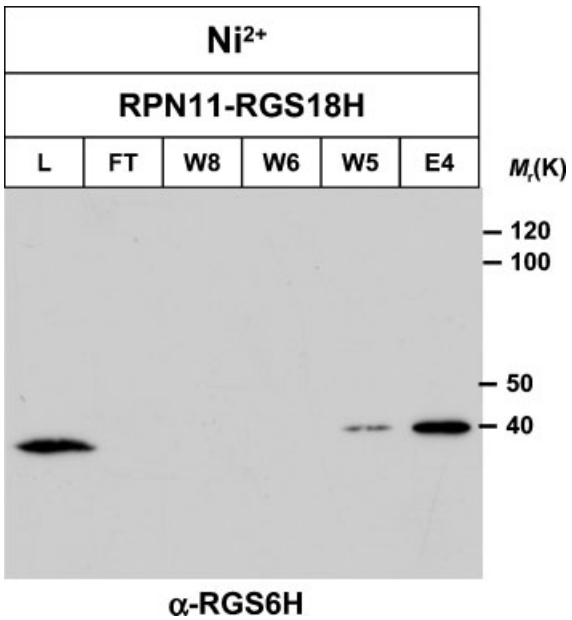

Figure 5. Ni^{2+} chelate chromatography of RGS18H-tagged Rpn11. Chromatography fractions were detected by immunoblotting with anti-RGS6H. Whole cell extract in 8 M urea buffers from cells expressing C-terminally RGS18H-tagged Rpn11 (L, load) was incubated with Ni^{2+} chelate resin. The unbound fraction (FT) showed close to 100% binding of $Rpn11^{RGS18H}$, further wash steps in 8 M urea at pH 8 (W8), pH 6.3 including 10 mM imidazole (W6) and pH 5 (W5) removed minimal amounts of bound protein. Recovery of $Rpn11^{RGS18H}$ by elution with pH 4.3 was complete (E4). Equivalent amounts were loaded for each fraction, so that the amount of detected $Rpn11^{RGS18H}$ can be directly compared

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