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# Chapter 4

## PCR-Mediated Epitope Tagging of Genes in Yeast

Radhika Mathur and Peter Kaiser

### Abstract

Epitope tagging of genes is a powerful technique facilitating assays for gene function, determination of subcellular distribution of proteins, affinity purification, study of protein interaction with other proteins, DNA or RNA, and any other antibody-based approach in the absence of protein-specific antibodies. Here, we describe a one-step PCR-based strategy for insertion of epitope tags at the chromosomal locus. This method takes advantage of efficient homologous recombination in yeast. PCR amplified tags are directed to desired chromosomal loci with the help of primer-encoded flanking homologous sequences enabling selective epitope tagging of genes of interest.

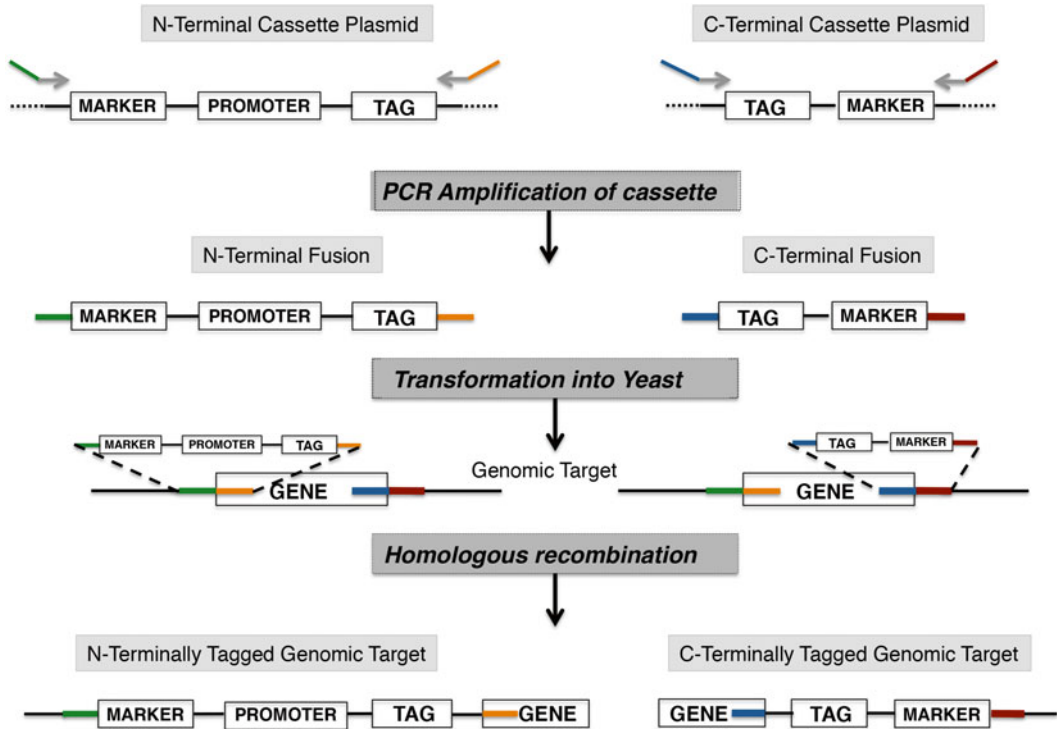
**Key words** *Saccharomyces cerevisiae*, Epitope tagging, Transformation, PCR-mediated gene modification

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### 1 Introduction

*Saccharomyces cerevisiae* is one of the most extensively studied eukaryotic model organisms in molecular and cellular biology. One of the characteristics that facilitate its use in rigorous genetic and biochemical analyses is highly active homologous recombination. A gene of interest can be efficiently and accurately replaced or tagged by transformation with a heterologous DNA fragment bearing as little as 40 nucleotides homology at its ends to the target sequence [1, 2].

Epitope tagging of open reading frames in yeast is a routine molecular biology procedure and is a valuable tool for studying proteins. The strategy requires a pair of primers that contain within their 5' region, around 40-nucleotides of homology to the genomic region of interest and around 20-nucleotide homology at their 3' end to the tag cassette (Fig. 1). A large array of cassette plasmids with a wide variety of tags (Table 1) in combination with different selection markers is now readily available for use in yeast [3, 4]. These plasmids mostly comprise of the same set of linker regions flanking the tag cassette such that a small set of primers can be used



**Fig. 1** The scheme of PCR-based epitope tagging

for an assortment of genome manipulations [3, 5]. Epitope tags are added either at the carboxyl (C) or amino (N) terminus of a protein. N-terminal tags typically rely on the use of heterologous promoters, though two-step strategies exist to tag genes at their N-termini under the control of their natural promoters [6].

Tag cassettes are PCR-amplified using these homology-bearing primers, and the PCR products are transformed directly into yeast. Transformants are selected based on the auxotrophic or antibiotic-resistance marker linked to the epitope tag. Successful epitope tagging is confirmed by immunoblotting to detect the protein of interest fused to the epitope tag.

## 2 Materials

Prepare all solutions in distilled water and store at room temperature, unless otherwise indicated.

### 2.1 PCR Amplification of Tag Cassette

1. TaKaRa ExTaq DNA Polymerase Kit (TaKaRa Bio Inc., Japan).
2. 3 M Sodium acetate, pH 5.2.
3. 100 % Ethanol (200 Proof).
4. 70 % Ethanol.

**Table 1**  
**List of tags available for epitope tagging in yeast**

3HA (Hemagglutinin)
GFP (S65T) green fluorescent protein
Ds-Red
yEGFP
ProA
TEV-ProA
TEV-ProA-7His
TEV-GST-6His
HBH (RGS6XHis-Biotin-6XHis)
HTB (RGS6XHis-TEV-Biotin)
Biotin
RGS18XHis
6XHis
S-tag
TAP
T7
Strep-tag II
FLAG
HSV
V5
VSV-G
GST (Glutathione S-transferase)
13Myc

Most plasmids cassettes with the above tags and various selection markers are available through Addgene ([www.addgene.org/](http://www.addgene.org/)) and EUROSCARF (<http://web.uni-frankfurt.de/fb15/mikro/euroscarf/plasmid.html>)

5. 1 % Agarose gel made in 1× TAE: 40 mM Tris acetate, 1 mM Ethylenediaminetetraacetic acid (EDTA).
6. 1× TE: 10 mM Tris–HCl, 1 mM EDTA, pH 7.5 (sterilized by autoclaving).

## **2.2 Yeast Transformation**

1. YEP medium: 1 % (w/v) yeast extract, 2 % (w/v) tryptone, 0.006 % (w/v) adenosine and uracil (sterilized by autoclaving).
2. 1 M Lithium acetate (LiOAc), pH 7.5 (filter sterilized).
3. 10× TE: 100 mM Tris–HCl, 10 mM EDTA, pH 7.5 (sterilized by autoclaving).

4. 62.5 % Polyethylene glycol 3350 (PEG) (filter sterilized).
5. Sheared salmon sperm DNA (10 mg/mL) Stored at  $-20^{\circ}\text{C}$ .
6. Dimethyl sulphoxide, >99.9 % purity.

### 2.3 Western Blotting

1. YEP media: 1 % (w/v) yeast extract, 2 % (w/v) tryptone, 0.006 % (w/v) adenosine and uracil (sterilized by autoclaving).
2. Urea Buffer: 8 M Urea, 300 mM NaCl, 100 mM Tris-HCl (pH 7.5), 0.2 % SDS, 10 mM sodium pyrophosphate, 5 mM EDTA, 5 mM EGTA, 50 mM NaF, 0.1 mM orthovanadate.
3. 100 mM Phenylmethanesulfonylfluoride (PMSF) in isopropanol. Should be stored at  $4^{\circ}\text{C}$ .
4. Protease Inhibitors: Aprotinin, Pepstatin, Leupeptin (each 1 mg/mL). Stored at  $4^{\circ}\text{C}$ .
5. Glass beads, 0.5 mm (Biospec Products, Inc., Bartlesville, OK).
6. Antifoam A (Sigma-Aldrich).
7. 4 $\times$  SDS sample buffer: 250 mM Tris-HCl pH 6.8, 8 % (w/v) SDS, 300 mM DTT, 30 % (v/v) glycerol, 0.02 % (w/v) bromophenol blue.
8. 0.45  $\mu\text{m}$  nitrocellulose PVDF membrane.
9. 10 $\times$  TBS-T (Tris-buffered saline with Tween): 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl pH 7.4, 0.2 % (v/v) Tween 20.
10. Blocking buffer: 5 % (w/v) nonfat dry milk in 1 $\times$  TBS-T.
11. 10 $\times$  TBS: 1.37 M NaCl, 27 mM KCl, 250 mM Tris-HCl pH 7.4.
12. 2 % Sodium azide.

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## 3 Methods

### 3.1 PCR Amplification of Tag Cassette

1. Perform PCR to amplify tag module using TaKaRa ExTaq DNA Polymerase Kit (*see Note 1*). Prepare a 300  $\mu\text{L}$  reaction containing 30  $\mu\text{L}$  10 $\times$  Polymerase Buffer, 2.5 mM  $\text{MgCl}_2$ , 1  $\mu\text{M}$  each primer, 0.2 mM each dNTP, 0.1  $\mu\text{g}$  plasmid DNA (containing the tag cassette) and 7.5 U of Taq DNA polymerase.
2. Divide the amplification mix from above into 3 or 6 PCR tubes with 100  $\mu\text{L}$  or 50  $\mu\text{L}$  each, respectively.
3. Use the following amplification conditions: Denaturation at  $94^{\circ}\text{C}$  for 5 min, followed by 5 cycles of  $94^{\circ}\text{C}$  for 1 min,  $45^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 2 min, and 22 cycles of  $94^{\circ}\text{C}$  for 1 min,  $60^{\circ}\text{C}$  for 1 min,  $72^{\circ}\text{C}$  for 2 min and conclude with an extension step at  $72^{\circ}\text{C}$  for 10 min (*see Note 2*).
4. Combine the PCR reactions into one microfuge tube (about 300  $\mu\text{L}$  total volume).

5. Analyze 5  $\mu\text{L}$  of PCR product by electrophoresis on a 1 % agarose gel to test efficiency of the PCR reaction.
6. Add 30  $\mu\text{L}$  3 M sodium acetate and 660  $\mu\text{L}$  100 % ethanol to the remaining PCR product and incubate at  $-20\text{ }^{\circ}\text{C}$  for 20 min to precipitate DNA (*see Note 3*).
7. Centrifuge in a microfuge at  $20,000\times g$  for 10 min to pellet DNA. Carefully decant supernatant. Add 1 mL 70 % ethanol. Mix and spin again briefly. Carefully decant supernatant. Air-dry the pellet for 10 min and dissolve PCR product in 20  $\mu\text{L}$   $1\times$  TE.

### 3.2 Yeast Transformation

This protocol has been adapted from [7] and [8].

All steps at room temperature unless otherwise stated.

1. Grow a starter culture of yeast overnight with shaking at  $30\text{ }^{\circ}\text{C}$  in 5 mL of appropriate media (*see Note 4*).
2. Next day, dilute the culture to  $A_{600}\sim 0.3$  to start 10 mL of secondary culture of yeast and grow to mid-log phase ( $A_{600}\sim 0.8$ ) (*see Note 5*).
3. Collect cells by centrifugation at  $2,000\times g$  for 3 min. Gently resuspend pellet in 2 mL LiOAc/ TE (10 mM Tris-HCl, 1 mM EDTA, 100 mM LiOAc) and then spin again for 3 min. Remove the buffer and gently resuspend cells in 100  $\mu\text{L}$  LiOAc/ TE and transfer to a fresh 1.5 mL centrifuge tube.
4. Add 5  $\mu\text{L}$  of denatured salmon sperm DNA (*see Note 6*) and 5  $\mu\text{L}$  of ethanol-precipitated PCR product from Subheading 3.1, step 7 (*see Note 7*). Gently mix by flicking the tube, and then incubate at room temperature without shaking for 5 min.
5. Prepare 280  $\mu\text{L}$  50 % PEG in LiOAc/TE solution for each transformation aliquot. For preparing 1 mL of this solution, add 100  $\mu\text{L}$   $10\times$  TE, 100  $\mu\text{L}$  1 M LiOAc, and 800  $\mu\text{L}$  62.5 % PEG.
6. Add 50 % PEG solution to the transformation reaction, mix by inverting the tube and incubate at  $30\text{ }^{\circ}\text{C}$  for 30–60 min without shaking (*see Note 8*).
7. Add 43  $\mu\text{L}$  DMSO and mix immediately by inverting the tube.
8. Heatshock in a water bath at  $42\text{ }^{\circ}\text{C}$  for 5 min.
9. Add 1 mL  $1\times$  TE and centrifuge at  $5,000\times g$  for 30 s to pellet the cells. Resuspend the cell pellet in 200  $\mu\text{L}$   $1\times$  TE and plate cells on selection plates (*see Note 9*).
10. Transformants usually appear after 2 days on plates incubated at  $30\text{ }^{\circ}\text{C}$ . Streak transformants onto fresh selection plates (*see Note 10*).

### 3.3 Western Blotting

To confirm successful epitope tagging 5–10 transformants should be analyzed by Immuno blotting. The rate of precise integration at the desired open reading frame varies depending on the locus, but is usually around 70 %. Any protein extraction and Western blotting

procedure will work to detect successful insertion of the epitope tag. Below we describe extraction using denaturing conditions, which preserves protein integrity better than many other protocols.

1. Grow transformants overnight with shaking in 1 mL of appropriate media. Also, use the parental strain without epitope tag as negative control sample. Next day, start 5–10 mL secondary culture of yeast and grow it up to  $A_{600} \sim 0.8$ . Collect cells by centrifugation ( $20,000 \times g$ , 1 min, room temperature), or by filtration. Quickly freeze the cell pellet and store it at  $-80^\circ\text{C}$  or process as below.
2. Lyse cell pellets in 200  $\mu\text{L}$  of Urea Buffer. Add protease inhibitors to buffer: 1 mM PMSF and 1  $\mu\text{g}/\text{mL}$  each—aprotinin, leupeptin, and pepstatin (*see Note 11*) with 200  $\mu\text{L}$  of glass beads and 1  $\mu\text{L}$  of Antifoam A. Perform lysis by rapid shaking using FastPrep FP120 (Qbiogene, Carlsbad, CA) at setting 4.5, three times for 40 s each, at  $4^\circ\text{C}$  (*see Note 12*).
3. Separate the lysate from glass beads (*see Note 13*). Centrifuge the lysate at  $20,000 \times g$  for 10 min at room temperature to remove cell debris and transfer the clarified supernatant to a fresh microfuge tube.
4. Quantify protein concentration and dilute samples to 4 M Urea using  $2 \times$  SDS sample buffer. Separate 20–40  $\mu\text{g}$  of protein lysate on a standard SDS polyacrylamide gel.
5. Transfer proteins onto PVDF membrane, block the membrane at room temperature with blocking buffer for 40 min with shaking and finally incubate the blot overnight with shaking at  $4^\circ\text{C}$  with appropriate antibody, diluted at manufacturer recommended dilution in blocking buffer with 0.02 % sodium azide (*see Note 14*).
6. Wash the blot twice with  $1 \times$  TBS-T (*see Note 15*) and then incubate with secondary antibody (diluted in blocking buffer according to manufacturer's recommendations) for 1 h at room temperature with shaking.
7. Wash the blot twice with  $1 \times$  TBS-T and twice with  $1 \times$  TBS and then proceed to developing the blot using a chemiluminescent substrate.

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## 4 Notes

1. Any other Taq polymerase kit can also be used.
2. The first set of PCR cycles at  $45^\circ\text{C}$  annealing temperature, ensures annealing of the short linker region of the primer to the tag cassette. The latter cycles at annealing temperature of  $60^\circ\text{C}$ , allow for optimal annealing of the whole primer to the generated PCR product template.

3. Precipitation of PCR DNA is an optional step but helps to increase the efficiency of yeast transformation.
4. Generally yeast strains should be grown in YEP media containing 2 % dextrose while strains requiring selection to retain plasmids should be grown in corresponding minimal media containing 2 % dextrose. Temperature-sensitive strains should be grown at room temperature.
5. For each transformation reaction, use 10 mL of yeast culture. Make sure to reserve one reaction for a control transformation.
6. To enhance transformation efficiency, heat sheared salmon sperm DNA in 100 °C water bath for 10 min and then put on ice before use.
7. A “no PCR” control transformation should also be performed with salmon sperm DNA but no PCR product.
8. To increase transformation efficiency, cells can be incubated at room temperature over night at this stage. Temperature-sensitive strains should be incubated at room temperature for a minimum of 60 min.
9. If you select for G418, hygromycin, or zeocin resistance, incubate cells in 1 mL YEP-Dex media for at least 4 h at 30 °C or at room temperature (for Zeocin selection incubate overnight at room temperature) to allow for expression of the selectable marker. If using microfuge tubes make sure to avoid popping of the caps due to CO<sub>2</sub> generation by growing yeast cells. Cap protector for microfuge tubes or screw-cap tubes can be used.
10. Antibiotic-containing plates can have high background from transiently transformed cells, containing nonintegrated PCR products. Replica plate the transformants onto a new selection plate after incubating for 2 days at 30 °C. Transiently transformed cells will no longer form colonies because nonintegrated PCR fragments are unstable. In some cases an additional round of replica plating after 24 h is necessary.
11. Urea buffer can be made before hand and stored at room temperature. PMSF and protease inhibitors should be added fresh, prior to use.
12. Cells can be vortexed with glass beads five times for 1 min at 4 °C, with a 1 min break on ice between the runs. Many other methods also exist for breaking yeast cells, such as TCA extraction, or alkali lysis.
13. A convenient way to separate the lysate from glass beads is to poke a hole at the bottom of the tube with a 21G needle, insert the tube with the hole into a fresh tube and centrifuge carefully for 30 s at 1,000 × *g*. The lysate will pass through the hole in the upper tube and collect in the lower tube, whereas the glass beads shall remain in the upper tube.



14. By adding sodium azide, one can increase the life of the diluted antibody. It can be used repeatedly and can be stored at  $-20^{\circ}\text{C}$  for a period of few months. Note that sodium azide should not be added to antibodies that are directly conjugated with HRP.
15. Each wash is performed for 10 min by shaking at room temperature.

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