



Yeast Functional Analysis Report

Modules for cloning-free chromatin tagging in *Saccharomyces cerevisiae*

Sabine Rohner, Susan M. Gasser* and Peter Meister

Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland

*Correspondence to:

Susan M. Gasser, Friedrich Miescher Institute for Biomedical Research, Maulbeerstrasse 66, CH-4058 Basel, Switzerland.
E-mail: susan.gasser@fmi.ch

Abstract

We describe a straightforward two-step PCR-based method to insert arrays of lac or tet operators (lacO or tetO) at specific loci in the budding yeast genome. The method entails insertion of a marker generated by PCR with classical long primers recognizing the locus of interest, followed by the replacement of this marker by a linearized plasmid bearing an array of lacI- or tetR-binding motifs. Using this technique, loci located either in the yeast genome or on yeast artificial chromosomes can be efficiently tagged. We provide a set of plasmids with different markers for cloning-free integration of lacO or tetO repeats into the yeast genome. Copyright © 2008 John Wiley & Sons, Ltd.

Keywords: nucleus; chromatin; tagging; lacO; tetO; CFC tags

Received: 30 September 2007
Accepted: 16 December 2007

Introduction

The study of chromatin organization in living budding yeast often relies on the recognition of integrated arrays of binding sites by fluorescently labelled bacterial DNA binding factors (reviewed in Hediger *et al.*, 2004; Neumann *et al.*, 2006). The binding sites consist of 128–256 repeats of the recognition consensus, which are generally cloned into a bacterial vector that also bears a yeast selection marker. The repetitive nature of these operators precludes the use of a PCR-based method for amplification or for the addition of sequences that provide homology for integration into specific genomic loci. Therefore, integration of lacO or tetO arrays at sites of interest has generally required the cloning of a small region of yeast DNA containing a unique restriction site into the array-containing plasmid, linearization of the plasmid at the unique cut site, and finally transformation with the linearized DNA. Unfortunately, repeated sequences are unstable in bacteria and the operator sites are often lost, due to recombination during the cloning process. To overcome the need for cloning, we present here a simple and versatile method for

cloning-free integration of arrays of lacO or tetO into the yeast genome.

Materials and methods

Construction of plasmids carrying target sequences for flanking markers in direct orientation

Target sequences for recombination-mediated integration were chosen based on their presence in most of the plasmids used for PCR-based marker integration (see sequences in Supplementary Figure 1). These sequences flank the selectable marker and can therefore be amplified at the same time as the desired marker, using primers described in Table 1. We first separately amplified target sequences by PCR with two sets of primers. This yielded the two desired DNA fragments and at the same time added *AscI* restriction sites to the outer sides of the fragments and two cloning sites to the inner side of the fragments (*AatII* and *KpnI*, respectively). Both fragments were cut with *AscI* and ligated. Ligated fragments were recovered before cloning with the TOPO TA Cloning kit

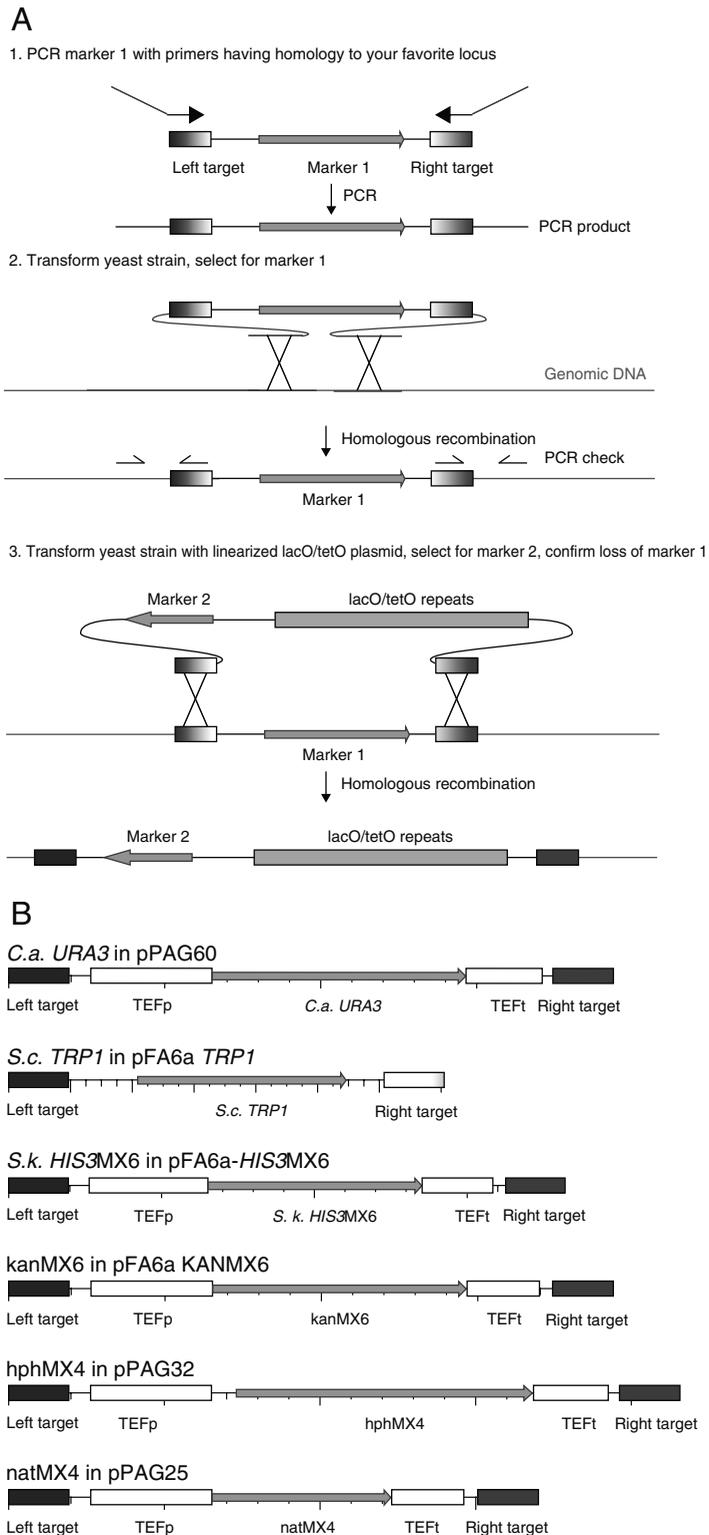


Figure 1. (A) Outline of the method for cloning-free chromatin tagging. (B) Previously available vectors for amplification of the first marker with target sequences (see text for explanations; for description of the markers, see Goldstein and McCusker, 1999; Longtine et al., 1998)

(Invitrogen). Recombinant plasmids were then cut with *Aat*II and *Kpn*I and cloned into vectors bearing lacO, tetO or the lacO/lexA repeats (pAFS52, pAFS59-TO and pFN15, respectively; Bystricky *et al.*, 2004; Hediger *et al.*, 2006; Straight *et al.*, 1996), using the same sites. All constructs were sequenced. These plasmids (pSR1, pSR3 and pSR2, respectively) do not contain a yeast selectable marker, and could be used for further cloning into pRS303 for *HIS3* addition; alternatively, *TRP1* or *LEU2* markers were added by PCR-mediated cloning (see Figure 2B for plasmid names and Supplementary Figure 1 for plasmid maps). For all cloning steps that involved lacO or tetO repeat-containing plasmids, the host bacteria (DH5 α) were grown at 30 °C to avoid excessive recombination and shrinkage of repeat length (Neumann *et al.*, 2006).

Table 1. PCR primers used for amplification of target-sequence flanked markers

Direct	5'–40-mer target locus–AGCGGATGCCGGGAGCAGAC
Reverse	5'–40-mer target locus–GTGAGCTGATACCGCTCGCC

PCR of first marker using long primers

Primers used were 60-mers, consisting of 40 nucleotides with homology to the target locus followed by a final 20 nucleotides that hybridize to the pUC18 backbone sequence of the marker plasmids, on either side of the marker (see Table 1). PCR was performed using PCR Master Mix (Qiagen) with 1 μ g marker plasmid as template and the following temperature programme: 95 °C for 4 min; (95 °C for 30 s, 52 °C for 1 min, 68 °C for 2.5 min) \times 5; (95 °C for 30 s, 58 °C for 1 min, 68 °C for 2.5 min) \times 25; 68 °C for 7 min); 5 μ l PCR reaction were used directly for yeast transformation without purification. After growth on selective medium, correct insertion was assayed using colony PCR.

Replacement of the first marker by lacO/tetO repeat plasmids

Plasmids (2–3 g/transformation) were cut with restriction enzyme *Asc*I (NEB) before direct transformation of yeast. After selection for the presence of the second marker, colonies were replica-plated on selective medium for the first marker to score for its loss and hence insertion of the repeats at the desired location.

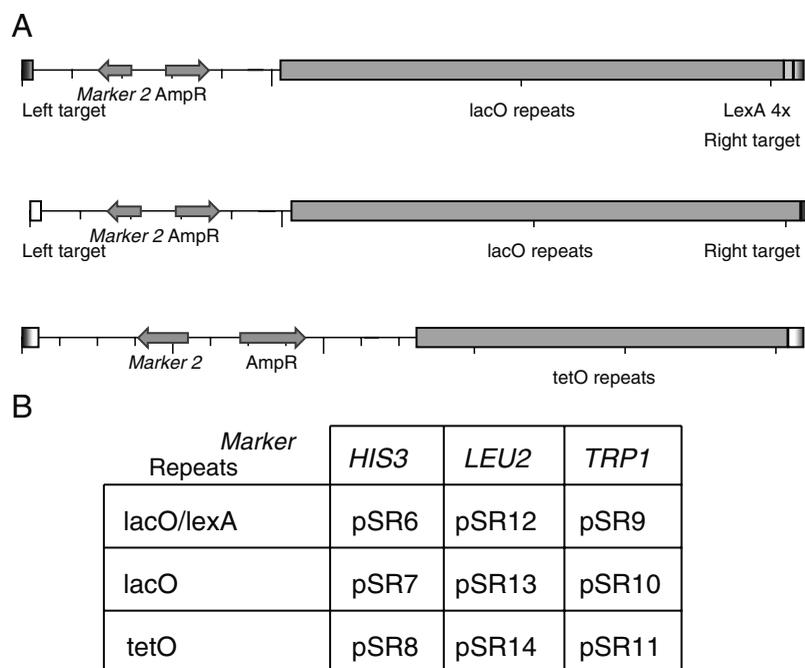


Figure 2. (A) Vectors for replacement of the first marker with lacO/lacO and lexA/tetO repeats. (B) Available plasmids for the second step of the cloning-free chromatin-tagging

Results and discussion

Our goal was to overcome the need for cloning and amplifying plasmids bearing arrays of bacterial repressor binding sites, in order to tag genomic loci with these repeats. To this end we developed a method that inserts such repeats without recloning them, through the use of a set of universal binding-array recombination cassettes. These can be introduced into the genome by homologous recombination with the common pUC18/19 backbone of the vectors widely used for gene deletions by PCR. The method involves two steps. First is the integration of a selectable marker gene at the locus of interest by homologous recombination in strains expressing the desired fluorescent DNA binding protein (Baudin *et al.*, 1993). For this, we modified the 3' ends of the long primers used for classical gene-targeting PCR, to allow amplification of pUC18/19 target sequences flanking the selectable marker gene (Figure 1A, steps 1 and 2; Table 1). Once the pUC18/19 plasmid target sequences, are integrated into the genome at your locus of interest, they can be used to integrate the selectable marker and lacO/tetO array cassette through a second step of homologous recombination.

The second step entails replacement of the first marker gene by the array of operators and a second selectable marker. The efficient recombination and 'popping out' of the first marker is achieved by direct pairing of the target sequences with the plasmid containing the repeats (also called 'ends-out configuration'). Therefore, the two 200 bp target sites were amplified by PCR and cloned in reverse orientation into plasmids containing the lacO or tetO repeats, separated by a unique restriction site, *AscI*. Subsequent linearization of the plasmid with *AscI* results in placement of the two target sequences on either side of the repeats in a direct orientation [Figure 1A (3)]. Alignment of these target sequences with the homologous sequences integrated into the genome during the first transformation leads to replacement of the first marker by the operator-containing plasmid [Figure 1A (3)].

Successful recombinants are recovered by plating the transformation on medium that selects for the second marker in the lac/tetO recombination cassette. The colonies are then replica-plated onto a medium that selects for the marker integrated in the first step. Transformants with integration at the

correct site will grow on the first but not on the second medium. Thereafter, it is highly recommended, although not absolutely necessary, to confirm correct integration of the array by PCR, because the probability of a concomitant gain of the second marker and loss of the first one by random events is extremely low. The second selection step is essential, however, because other plasmids that might have been introduced previously into the recipient yeast strain may carry the target sequences for the operator-containing plasmid.

As in the cloning-based methods used until now, our method results in the insertion of a single marker associated with the operator array. The development of lac/tetO recombination cassettes that exploit marker genes from organisms other than *S. cerevisiae* might reduce further the chances for homologous recombination at sites other than the pUC18/19 target sequences.

It is still necessary to evaluate the number of operators integrated into the genome, since repeats are frequently recombined out by homologous recombination in the yeast (described in detail in Neumann *et al.*, 2006). A rough quantitation can be done by microscopic observation, simply by comparing the intensity and size of the GFP–LacI focus with the GFP–Nup49 nuclear pore marker (Hediger *et al.*, 2004; Neumann *et al.*, 2006). When comparing the intensity and size of the GFP–lacI or tetR–GFP foci, we did not see differences in the size or intensity of the spot in strains created by the standard subcloning method or by this recombination/marker replacement method (data not shown).

The new method presented here has the advantage of avoiding the insertion of additional direct repeats flanking the array of operators, which increases the risk that the array might be lost by recombination. We tested our system by inserting lacO or tetO repeats at a telomeric locus and at internal sites within yeast artificial chromosomes (Wormbase Y6G2 and Y97B12) carrying *Caenorhabditis elegans* DNA. Efficiencies of the replacement varied depending on the locus, yet 10–30% of the colonies which were auxotroph for the second marker had lost the first one (see Table 2 for replacement efficiencies of target sequences introduced with a *URA3* marker by plasmid pSR6; similar results were obtained with the other plasmids). When these colonies were tested

Table 2. Typical replacement efficiency obtained with a target-sequence flanked *C. albicans* *URA3* marker by pSR6 transformation (3 µg Ascl-digested DNA)

Strain/target locus	Colonies positive for the second marker (HIS ⁺)	Colonies positive for second marker with loss of first marker (URA ⁻ HIS ⁺)	Ratio (%)
GA-4132/TelVIII	47	13	27
GA-4219/Y97B12 YAC	478	103	22
GA-4262/Y6G2 YAC	626	157	25

microscopically, at least 1 of 8 colonies tested by microscopy showed a bright spot.

The cloning-free method described here is very flexible in terms of markers used for the first and the second step. For the first step, useful markers include the standard markers such as *Candida albicans* *URA3*, *S. cerevisiae* *TRP1*, *Saccharomyces kluyveri* *HIS3*, as well as dominant drug-resistance cassettes, kanMX6, hphMX4 or natMX4 (Figure 1B). Since the target sequences can be found flanking the marker in many other plasmids, it is likely that other selectable genes might also be used. For the second step, we created plasmids carrying the *TRP1*, *LEU2* or *HIS3* markers. For each of these markers, three repeat types are available; arrays of lacO, tetO or lacO with four lexA sites for use in assays that require protein targeting (Figure 2A, B, and Supplementary Figure 1). These plasmids are available upon request, together with their full sequences.

Acknowledgements

We thank the Gasser laboratory for helpful discussions and comments on the system. We are grateful to Veronique Kalck for excellent technical assistance. We thank Primo Schär for critical reading of the manuscript. This work was supported by the EU NOE 'Epigenome', the Swiss National Science Foundation NCCR programme 'Frontiers in Genetics' and the Novartis Research Foundation.

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