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High-efficiency Gene Targeting in *Schizosaccharomyces pombe* Using a Modular, PCR-based Approach with Long Tracts of Flanking Homology

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Abstract

Bähler *et al.* (1998) recently described a PCR-based system for the deletion, tagging and overexpression of endogenous genes in the fission yeast *Schizosaccharomyces pombe*. A small set of PCR primers can be used to generate gene-targeting substrates from each of several modules that differ in the selectable marker (*ura4⁺* or *kanMX6*), the presence or absence of specific epitope tags (HA, Myc, GST or GFP), the position in which the epitopes will be inserted (C- or N-terminal), and the presence or absence of a regulatable promoter (the *nmt1* promoter). This is a straightforward and powerful system: nine different genes were C-terminal tagged at an average efficiency of 73%, using primers producing only 60–81 bp of homology. In contrast, when studying three transcriptionally-silent genes (*rec8⁺*, *rec10⁺* and *rec11⁺*) we obtained an average homologous integration efficiency of 4% for 12 targeting constructs when using primers that contained 80 bp of homology. By using a PCR-based increase in the amount of flanking homology to ≥ 250 bp, we obtained homologous integration efficiencies of up to 100%. Thus, loci of *S. pombe* that are refractory to gene targeting when using short tracts of homology can be readily modified by increasing the extent of homology flanking the targeting modules. This straightforward and cost-effective approach might therefore be the one of choice for the modification of *S. pombe* loci in general and of targeting-refractory loci in particular.

Keywords

fission yeast; polymerase chain reaction; homologous recombination; targeted integration; epitope tagging; gene disruption; gene expression

INTRODUCTION

In fission yeast, as in budding yeast, variations of the one-step gene disruption method provided a first generation of tools for the functional analysis of endogenous genes (Rothstein, 1983; Grimm and Kohli, 1988; Grimm *et al.*, 1988; Baudin *et al.*, 1993; Grallert *et al.*, 1993; Keeney and Boeke, 1994). Coupled with *in vitro* recombinant DNA technology, this approach also allows researchers to modify genes in various ways beyond the construction of simple deletions. For example, genes can be manipulated so as to encode fusion proteins harbouring epitope tags (Kolodziej and Young, 1991; Prasher, 1995).

A second generation of tools for endogenous gene manipulation has been developed based upon the observation that PCR products that terminate in relatively short stretches of homology (provided by the PCR primers) frequently yield transformants that have targeted to the locus of interest (Baudin *et al.*, 1993; Wach *et al.*, 1994; Kaur *et al.*, 1997; Wach *et al.*, 1997; Bähler *et al.*, 1998; Longtine *et al.*, 1998). This permits the construction of deletions, replacement of endogenous promoters with regulatable promoters and tagging of gene products without any cloning steps. What makes the approach particularly advantageous is that a variety of different modules can each be amplified using a small set of PCR primers (Bähler *et al.*, 1998; Longtine *et al.*, 1998), thereby providing a versatile and efficient toolkit.

An example of the modular, PCR-based targeting approach to constructing genes encoding proteins with a C-terminal epitope tag is shown in Figure 1. In essence, one uses hybrid primers that contain homology to the target gene of interest and to a modular plasmid template. After PCR amplification of the module, the PCR product is transformed into yeast. Stable transformants are selected and a screen is applied to identify those that have successfully targeted the construct to the locus of interest by homologous recombination. The basic template module encodes an epitope destined to be linked in-frame to the gene of interest and a complete expression cassette containing a selectable marker gene. Two PCR primers can be used to generate gene-targeting substrates from each of several different modules (Bähler *et al.*, 1998; Longtine *et al.*, 1998). The modules encode three copies of the influenza haemagglutinin (HA) epitope (Field *et al.*, 1988; Tyers *et al.*, 1992), 13 copies of the human *c-myc* (Myc) epitope (Evan *et al.*, 1985; Munro and Pelham, 1987), glutathione *S*-transferase (GST) from *Schistosoma japonicum* (Smith *et al.*, 1986), or the green fluorescent protein (GFP) from the jellyfish *Aequorea victoria* (Prasher, 1995). The HA and Myc epitopes are recognized by commercially-available antibodies, proteins with the GST epitope may be affinity purified with glutathione beads, and proteins with the GFP epitope can be visualized by fluorescence microscopy or with antibodies.

The *rec8⁺*, *rec10⁺* and *rec11⁺* genes of *S. pombe* are expressed only in meiosis (Lin *et al.*, 1992; Lin and Smith, 1995; Li *et al.*, 1997) and define a pathway of meiotic chromosome dynamics (Molnar *et al.*, 1995; M.D. Krawchuk, L.C. DeVeaux, and W.P. Wahls, 1999). We used the modular, PCR-based, gene-targeting system (Bähler *et al.*, 1998; Longtine *et al.*, 1998) to try to modify these genes so that they expressed proteins with C-terminal epitope tags. Surprisingly, we found that these three genes were refractory to gene targeting when using short (80 bp) tracts of homology. We then used a PCR-based approach to increase the extent of homology. This markedly increased the efficiency of gene targeting and may therefore be useful for the modification of other *S. pombe* loci.

MATERIALS AND METHODS

PCR amplification of gene-targeting modules with short (80 bp) tracts of flanking homology

Unless otherwise indicated, the oligonucleotides used as primers (Table 1) were synthesized and PAGE-purified by Integrated DNA Technologies. Plasmid substrates were pFA6a-3HA-kanMX6, pFA6a-13Myc-kanMX6, pFA6a-GST-kanMX6, pFA6a-GFP-kanMX6, pYL3 (*rec8⁺*), and pYL150 (*rec10⁺*) (Lin *et al.*, 1992; Lin and Smith, 1995; Wach *et al.*, 1997; Bähler *et al.*, 1998; Longtine *et al.*, 1998). The *rec11⁺* gene was obtained by PCR directly from *S. pombe* genomic DNA. Because there are multiple errors in the published coding sequence of *rec8⁺* (Lin *et al.*, 1992), we used the corrected *rec8⁺* coding sequence (GenBank AJ223299), kindly provided by Jürg Kohli. Gene-targeting fragments with short (80 bp) tracts of flanking homology were generated by PCR, as described (Bähler *et al.*, 1998), with the following modifications. To optimize the processivity and fidelity of the

PCR extensions, we used a mixture of *Taq* and *Pfu* polymerases (15:1). Eight duplicate reactions were conducted for each amplification to reduce further the likelihood of deleterious 'jackpot' mutations. Each PCR reaction was in a volume of 25 μ l and contained 5 units of polymerase. Duplicate reaction products were pooled and gel-purified prior to transformation.

PCR amplification of gene-targeting modules with long (≥ 250 bp) tracts of flanking homology

The oligonucleotide primers (Table 1) were synthesized and desalted by Bethesda Research Laboratories. The first round of PCR amplification was used to obtain two DNA molecules per target locus, one to the left of the target site and one to the right, each ≥ 250 bp in length. One primer in each reaction was designed as a hybrid primer that also contained homology to one of the primer binding sites in the pFA6a modules (Table 1).

First-round PCR reactions were in a volume of 25 μ l and contained 2.5 μ l of PCR buffer (20 mM Tris-HCl, pH 8.8, 500 mM KCl, 150 μ g/ml bovine serum albumin, 25 mM MgCl₂), 50 μ M of each dNTP, 2 μ M of each primer, 5 units of *Taq:Pfu* polymerases 15:1, and 50 ng plasmid or 1 μ g genomic template DNA. Eight duplicate PCR reactions were carried out for each template/primer set to reduce the likelihood of 'jackpot' mutations. Thirty cycles of PCR were carried out (94°C for 30 s, 55°C for 30 s, and 72°C for 2 min). The final 72°C cycle was extended by 5 min. The duplicate reactions were pooled, extracted with an equal volume of phenol:chloroform (1:1), 0.25 volumes of 8M NH₄OAc and 2.5 volumes of ethanol were added, and the DNA samples were precipitated by centrifugation at 15 000 $\times$ g for 30 min. The supernatants were discarded, the pellets air-dried, and each DNA sample resuspended in 200 μ l of TE. The PCR products were resolved by preparative electrophoresis in 1.2% agarose gels containing 1 μ g/ml of ethidium bromide. Products were visualized with a low-power, hand-held, long-wave UV light source (FB-UVLS80, Fisher Biotech) and the desired bands were excised. The DNA samples were recovered by electroelution into TAE buffer within dialysis tubing, were extracted serially with equal volumes of phenol, phenol:chloroform (1:1) and chloroform, and were recovered by ethanol precipitation, as described above. The DNA samples were resuspended in 25 μ l of TE. Each pooled sample typically yielded 1–5 μ g of DNA.

The second round of PCR was used to attach the ≥ 250 bp blocks of homology to each of the four pFA6a plasmid modules. A mixture of the left and right PCR products from the first round of PCR, the desired pFA6a template module, and an excess of the two outermost PCR primers were used to generate gene-targeting fragments flanked on each side by long tracts of homology to the target loci.

Second-round PCR reactions were in a volume of 25 μ l and contained 2.5 μ l of PCR buffer (described above), 50 μ M of each dNTP, 2 μ M of each primer, 50 ng of each left and right PCR product from the first round of PCR, 5 units *Taq:Pfu* polymerases 15:1, and 50 ng of pFA6a template DNA. Eight independent PCR reactions were carried out for each template/primer set. Five initial cycles of PCR were conducted at low stringency (94°C for 10 s, 40°C for 30 s, and 68°C for 4 min) and were followed by 20 cycles at higher stringency (94°C for 10 s, 55°C for 30 s, and 68°C for 4 min). The final 68°C cycle was extended by 5 min. Duplicate products were pooled and gel-purified, as described above. The DNA fragments were further purified by serial extraction with equal volumes of phenol, phenol:chloroform (1:1) and chloroform, then ethanol-precipitated and resuspended in 25 μ l of TE. Each pooled sample typically yielded 5–10 μ g of DNA.

Transformation of cells and screening of stable transformants for those with homologous integration

S. pombe strain WSP 20 (h^+ *ade6-M26 pat1-114^{ts}*) was used for transformations. Each transformation contained approximately 10 μ g of the gene-targeting DNA fragment. Unless otherwise indicated in the text, we followed the transformation protocol of Keeney and Boeke (1994), as described by Bähler *et al.* (1998). Selection for stable G418-resistant colonies and PCR diagnostic analysis of whether the transformants resulted from homologous gene targeting, or non-homologous, random integration, were as described (Bähler *et al.*, 1998), using locus-specific primers flanking the target sites.

RESULTS AND DISCUSSION

Low efficiency of gene targeting of the *rec8⁺*, *rec10⁺* and *rec11⁺* loci when using 80 bp of flanking homology

To construct *rec8⁺*, *rec10⁺* and *rec11⁺* genes expressed at their endogenous loci, under regulation of their own promoters, and encoding proteins with C-terminal epitope tags, we used the protocol of Bähler *et al.* (1998) (Figure 1). For each gene we constructed one set of hybrid primers (Table 1) and we used those primers to generate gene-targeting fragments that contained 80 bp of homology to the target locus on each side of the pFA6a modules (Figure 1). For each locus we generated gene-targeting fragments designed to integrate and express each of four different C-terminal epitope tags, 3 $\times$ HA, 13 $\times$ Myc, GST, and GFP. The gene-targeting fragments were gel-purified and used in parallel to transform *S. pombe* cells. Transformants that were resistant to G418 upon initial selection and subsequent culture were subjected to further analysis.

The G418-resistant colonies could have stably incorporated the transforming DNA into their genomes, either by homologous recombination with the *rec8⁺*, *rec10⁺* and *rec11⁺* target loci (Figure 1) or by non-homologous integration of the transforming DNA elsewhere in the genome. To distinguish between the two classes, genomic DNA from the transformants was used as the template for PCR amplification, using locus-specific primers flanking the site to which each particular gene-targeting fragment was directed. The data are given in Table 2. For many of the 12 different configurations (four different epitope constructs for each of the three different *rec⁺* genes), we failed to obtain any targeted integrants and the average homologous integration efficiency for all 12 was 4%. This value is markedly different from the average homologous integration efficiency of 73% obtained when using the identical procedure on nine different genes (Bähler *et al.*, 1998).

The gene-targeting method that we used is thought to depend upon a high quality of the long (~100 nucleotides) hybrid primers used, achieved by a combination of high fidelity synthesis and by polyacrylamide gel electrophoresis purification (Bähler *et al.*, 1998; Longtine *et al.*, 1998). We therefore tested whether primers obtained from different sources and gel-purified by the supplier or within our laboratory exhibited any differences in their utility for homologous gene targeting. In each case we obtained similar, very low efficiencies of homologous integration at the *rec8⁺*, *rec10⁺* and *rec11⁺* loci (data not shown). The simplest interpretation is that the *rec8⁺*, *rec10⁺* and *rec11⁺* loci are refractory to homologous gene targeting when using short (80 bp) tracts of homology, perhaps because these genes are not expressed in mitotic cells (Lin *et al.*, 1992; Lin and Smith, 1995; Li *et al.*, 1997). Alternatively, gene targeting with short lengths of flanking homology in *S. pombe* might be extremely sensitive to experimental parameters beyond our control.

Enhancement of gene-targeting efficiencies by a PCR-based increase in the amount of flanking homology to ≥ 250 bp

In *S. pombe*, homologous integration efficiencies of 1–3% have been obtained with about 40 bp of flanking homology (Kaur *et al.*, 1997), efficiencies of $\geq 29\%$ have been obtained with about 80 bp of flanking homology (Bähler *et al.*, 1998), and efficiencies of $\geq 50\%$ are routinely obtained with ≥ 500 bp of flanking homology (Grimm and Kohli, 1988; Grallert *et al.*, 1993; Keeney and Boeke, 1994). We therefore used a PCR-based strategy that could increase the amount of homology to the target locus on each side of the gene-targeting fragment.

The approach to increase the amount of flanking homology is based upon ‘recombinant PCR’ methods often used for mutagenesis applications (Higuchi, 1990; Wach, 1996). In essence, one round of PCR was used to generate ≥ 250 bp of homology on each side of the desired target site, and then a second round of PCR was used to attach those blocks of homology to the pFA6a module of interest (Figure 2). For each locus we generated gene-targeting fragments designed to express each of four different C-terminal epitope tags, 3 $\times$ HA, 13 $\times$ Myc, GST, and GFP. The gene-targeting fragments were gel-purified and used in parallel to transform *S. pombe* cells. Stable, G418-resistant transformants were obtained at frequencies similar to those obtained when using shorter (80 bp) tracts of flanking homology.

We used diagnostic, locus-specific PCR to determine the relative homologous integration efficiency for each of four modules targeted to each of the three *rec*⁺ genes (Table 2). In contrast to the results using short (80 bp) tracts of homology, many of the transformants obtained using the longer (≥ 250 bp) tracts of homology were found to be targeted to the loci of interest (Table 2). The homologous integration efficiencies for the 12 different targeting constructs ranged from 20% to 100% and the overall efficiency was 58%. We conclude that loci that are refractory to gene targeting when using short tracts of homology, such as the transcriptionally silent *rec8*⁺, *rec10*⁺ and *rec11*⁺ genes, can be readily modified by a simple procedure that increases the extent of homology flanking the targeting modules.

Confirmation of successful target locus modification

Expression of the epitope-tagged versions of the wild-type genes fully complemented the meiotic recombination defects of their respective mutants and also produced meiotically-induced polypeptides of the expected sizes that were detectable by Western blotting (data not shown). We conclude that the modified gene-targeting procedure can generate fusion proteins that are biologically functional and that can be detected with immunological reagents.

Cost–benefit comparison of modular PCR-based gene targeting with short and long tracts of flanking homology

The gene-targeting approach using about 80 bp of homology flanking each side of the targeting fragment is straightforward and rapid (Figure 1) (Bähler *et al.*, 1998; Longtine *et al.*, 1998). Essentially, one round of PCR is followed by transformation, diagnostic PCR analysis, and subsequent analysis of the expressed fusion protein. A single pair of hybrid oligonucleotides (Table 1) can be used to amplify fragments from any of the modular pFA6a templates, permitting a variety of different tags to be incorporated in-frame at the desired target locus. The two hybrid primers are each about 100 nucleotides long and must be gel-purified. The cost for a pair of such oligo-nucleotides is on the order of \$300. While many loci can be readily targeted using short flanking homology (Bähler *et al.*, 1998), many other loci may be refractory to this approach (Table 2).

The modified approach producing longer tracts of flanking homology can also be used for the modular targeting (Figure 2). The principal difference is that this approach utilizes a set of four oligonucleotide primers (Table 1) and two rounds of PCR amplification. Two of the primers are approximately 50 nucleotides in length, two others are approximately 20 nucleotides in length, and there is no need for gel purification; desalted oligonucleotides are simply dissolved in buffer and used directly. The cost for a set of such oligo-nucleotides is in the order of \$150. The additional PCR step increases the time required for gene-targeting fragment preparation by one or two days and, as with all PCR applications, may require additional optimization of amplification parameters. However, the parameters (e.g. reaction conditions, product length, etc.) are similar in both approaches and we had no difficulty in obtaining functional gene-targeting fragments containing ≥ 250 bp of flanking homology for each of the three genes tested (Table 2).

Conclusions

Some loci of *S. pombe* are refractory to gene targeting when using short tracts of homology. Such loci can be readily modified by using a PCR-based increase in the extent of flanking homology. Since producing gene-targeting substrates with longer tracts of homology is facile, cost-effective and still takes advantage of the benefits of the modular system (Bähler *et al.*, 1998), this approach might be the one of choice for the modification of loci in general and of targeting-refractory loci in particular.

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Figure 1.

Use of heterologous modules for PCR-based gene targeting in *S. pombe*. The strategy for placing an epitope tag at the C-terminal codon of a gene is shown, but the same type of approach can be used for N-terminal tagging or gene disruption (Bähler *et al.*, 1998). The schematic diagrams are not to scale. (A) The pFA6a plasmid derivatives (Wach, 1996; Bähler *et al.*, 1998; Longtine *et al.*, 1998) contain two primer binding sites (PBS). PBS 1 encodes a linker polypeptide that is in-frame with one of several modules encoding various different epitope tags. A transcription terminator (term.) is included in most constructs. Also included between the two PBS is a selectable marker cassette containing either the *S. pombe ura4⁺* gene or the *kanMX6* gene and all the regulatory sequences required for expression in *S. pombe*. Hybrid PCR primers are designed to contain approximately 80 nucleotides of homology to the target locus and 20 nucleotides of homology to the PBS. The left-most primer is designed to place the 3' end of the target locus coding region in-frame with PBS 1 and the epitope module. Because of the modular nature of the pFA6a templates, a single pair of gene-specific primers may be used for each of the modules. (B) PCR amplification generates a gene targeting fragment flanked on each side by about 80 bp of homology to the target locus. (C) Upon transformation, some of the DNA fragments align with the target locus by virtue of this flanking homology. (D) Homologous gene targeting and non-

homologous, random integration (not shown) can each give rise to selectable transformants. The type of integration may be readily distinguished by PCR analysis of DNA from transformants. (E) Expression of the modified locus from its endogenous promoter produces a polypeptide that contains the desired epitope tag on its C-terminus.

Figure 2.

Strategy for a PCR-based increase in the amount of flanking DNA with homology to the target locus. The schematic diagrams are not to scale. (A) Two PCR reactions are configured to amplify independently ≥ 250 bp of the target locus flanking the desired site of integration. In each case, one of the primers (*w* or *z*) is homologous only to the target locus and one of the primers is a hybrid primer containing both gene-specific (*x* or *y*) and pFA6a-specific (*pFA6a-1'* or *pFA6a-2'*) DNA sequence homology. One of the primers (*x*, *pFA6a-1'*) is designed to place the 3' end of the target locus coding region in-frame with PBS 1 and the epitope module. (B) The two independent PCR reactions generate long blocks of homology to the target locus ('left PCR product' and 'right PCR product'). (C) The left and right PCR products are subsequently mixed with pFA6a template and an excess of the two outermost, locus-specific PCR primers (*w* and *z*). (D) PCR amplification generates a gene targeting fragment flanked on each side by ≥ 250 bp of homology to the target locus. Transformation and screening of transformants is then as described in Fig. 1C–E.

Table 1

PCR primers.

Locus (position) ^a	Oligonucleotide sequence	
For the generation of 80 bp of flanking homology (Figure 1)		
	Locus-specific sequence ^b	pFA6a-specific sequence ^c
<i>rec8</i> ⁺ (a, pFA6a-1)	5'-AG TCT GCA TT ... C GAT GCC ATT	CGG ATC CCC GGG TTA ATT AA-3'
<i>rec8</i> ⁺ (b, pFA6a-2)	5'-ATTATTTTGA ... GTGAAAAAGTT	GAATTCGAGCTCGTTTAAAC-3'
<i>rec10</i> ⁺ (a, pFA6a-1)	5'-CT AGA TAT AA ... G ATG TTT AAG	CGG ATC CCC GGG TTA ATT AA-3'
<i>rec10</i> ⁺ (b, pFA6a-2)	5'-TAAACGCTTCC ... TAGGTTCAAG	GAATTCGAGCTCGTTTAAAC-3'
<i>rec11</i> ⁺ (a, pFA6a-1)	5'-GG TAT CCC AA ... C CAT TTA TCA	CGG ATC CCC GGG TTA ATT AA-3'
<i>rec11</i> ⁺ (b, pFA6a-2)	5'-CCTTGCTCTCA ... AAATTTCGTT	GAATTCGAGCTCGTTTAAAC-3'
For the generation of ≥250 bp of flanking homology (Figure 2)		
	pFA6a-specific sequence ^c	Locus-specific sequence ^b
<i>rec8</i> ⁺ (w)	5'-	TCCTGTGACTGACTTCGTAC-3'
<i>rec8</i> ⁺ (pFA6a-1', x)	5'-GGG GAT CCG TCG ACC TGC AGC GTA CGA	AAT GGC ATC GGT GCT TTT T-3'
<i>rec8</i> ⁺ (pFA6a-2', y)	5'-GTTTAAACGAGCTCGAATTTCATCGAT	AAC TTTTCACATGTTAAACT-3'
<i>rec8</i> ⁺ (z)	5'-	CTGACTAACAAAGACTCGT-3'
<i>rec10</i> ⁺ (w)	5'-	ACTAGATGGTACCTGCTC-3'
<i>rec10</i> ⁺ (pFA6a-1', x)	5'-GGG GAT CCG TCG ACC TGC AGC GTA CGA	CTT AAA CAT CAA ACT GTC A-3'
<i>rec10</i> ⁺ (pFA6a-2', y)	5'-GTTTAAACGAGCTCGAATTTCATCGAT	CTTGAACCTATAGTATCAAA-3'
<i>rec10</i> ⁺ (z)	5'-	CCGTACTGCTGCTGAGGA-3'
<i>rec11</i> ⁺ (w)	5'-	GCTAAGGTTGCGAAGCAA-3'
<i>rec11</i> ⁺ (pFA6a-1', x)	5'-GGG GAT CCG TCG ACC TGC AGC GTA CGA	TGA TAA ATG GTC AGC TGT T-3'
<i>rec11</i> ⁺ (pFA6a-2', y)	5'-GTTTAAACGAGCTCGAATTTCATCGAT	AACGAAATTTTAAACACTAAT-3'
<i>rec11</i> ⁺ (z)	5'-	-ccATCCAACTGGCTACGATC-3' ^d

^aThe positions indicated in parentheses correspond to the primer positions shown schematically in Figures 1 and 2. pFA6a-1' is essentially the reverse complement of pFA6a-1 and pFA6a-2' is essentially the reverse complement of pFA6a-2.

^bThe ellipses represent 60 nucleotides of sequence not shown. The reading frame for each locus-specific sequence (a, b, w, x, y, or z in Figures 1 and 2) is shown only if it must be fused in-frame to the pFA6a module. In those cases, the last codon of the locus-specific sequence in the primer (or of its reverse complement) corresponds to the C-terminal codon of the locus. Locus-specific sequences in primers corresponding to the right side of the target loci (b or y in Figures 1 and 2) were designed to anneal downstream of the stop codon. Locus specific primers w and x were selected with the aid of a

computer program designed to identify the optimal primers (PrimerSelect, DNASTAR, Inc.) and were positioned to permit amplification of ≥ 250 bp blocks of flanking homology when used in combination with their respective target site primers (Figure 2).

^cThe reading frames for the pFA6a-1 and pFA6a-1' primers are shown because they must be fused in-frame to the locus-specific sequences in order to generate a PCR product encoding the fusion protein.

^dThis oligonucleotide inadvertently contained on its 5' end a two bp mismatch (lowercase) to the target locus (Li *et al.*, 1997), but the substitution was fortuitously positioned, such that it did not adversely affect the PCR extension or the desired outcome of the targeting.

Table 2

Efficiency of homologous integration when using short (80 bp) or long (≥ 250 bp) tracts of PCR-generated homology flanking the targeting module.

Gene	C-terminal tag	Length of homology ^a	Positives/total tested ^b	Homologous efficiency ^c (%)
<i>rec8</i> ⁺	3×HA	80 and 80	2/15	13
<i>rec8</i> ⁺	13×Myc	80 and 80	0/29	0
<i>rec8</i> ⁺	GST	80 and 80	0/10	0
<i>rec8</i> ⁺	GFP	80 and 80	1/38	2.6
<i>rec10</i> ⁺	3×HA	80 and 80	0/2	0
<i>rec10</i> ⁺	13×Myc	80 and 80	0/6	0
<i>rec10</i> ⁺	GST	80 and 80	0/0	0
<i>rec10</i> ⁺	GFP	80 and 80	0/2	0
<i>rec11</i> ⁺	3×HA	80 and 80	1/21	4.8
<i>rec11</i> ⁺	13×Myc	80 and 80	0/13	0
<i>rec11</i> ⁺	GST	80 and 80	2/10	20
<i>rec11</i> ⁺	GFP	80 and 80	1/13	7.7
<i>Total</i>			7/159	4.4
<i>rec8</i> ⁺	3×HA	333 and 285	22/32	69
<i>rec8</i> ⁺	13×Myc	333 and 285	10/33	30
<i>rec8</i> ⁺	GST	333 and 285	10/17	59
<i>rec8</i> ⁺	GFP	333 and 285	5/9	56
<i>rec10</i> ⁺	3×HA	442 and 553	10/21	48
<i>rec10</i> ⁺	13×Myc	442 and 553	8/15	53
<i>rec10</i> ⁺	GST	442 and 553	2/10	20
<i>rec10</i> ⁺	GFP	442 and 553	10/11	91
<i>rec11</i> ⁺	3×HA	450 and 516	7/7	100
<i>rec11</i> ⁺	13×Myc	450 and 516	9/16	56
<i>rec11</i> ⁺	GST	450 and 516	8/10	80
<i>rec11</i> ⁺	GFP	450 and 516	10/10	100
<i>Total</i>			111/191	58

^a Number of bp in the gene targeting fragment with homology to the target locus, left and right of the pFA6a module.

^b Stable G418-resistant transformants were screened to determine which resulted from homologous gene targeting. The data are the number due to homologous integration/the total number of stable transformants screened.

^c Percentage of stable transformants arising by homologous integration at the desired target locus.