Cas9-mediated gene editing in Saccharomyces cerevisiae

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Method Article

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Abstract

We describe an easy, efficient, and inexpensive way to clone gRNAs into plasmid vectors carrying Cas9. The method involves designing two 25 nt complementary sequences that can be duplexed and subsequently ligated into the BpII restriction site of the plasmid vector. Using the above method, we have been able to introduce point mutations and deletions in varying sizes by using 80-nt single-stranded DNA, PCR products or gBlocks as templates.

Introduction

Protocol for gene targeting with Cas9

Reagents

BpII enzyme \(from Thermo Fisher) Ligase \(from NEB)

Procedure

I) Digesting the plasmid with BpII: 66µl TE 20µl plasmid \(~ few µg) from a standard miniprep that has a yield of 50-100ng/µl 10µl Tango buffer \(provided with BpII) 2µl 50x SAM \(provided with BpII) 2µl BpII enzyme 100µl total Incubate overnight at 37C. After the digest, gel purify the cut plasmid. II) duplexing the oligos: a) Order the oligos the following way: Example target sequence: 5'CCCAGGTATTGTTAGCGGTTTGAACGCTGCAGG 3' Select the 20 nt sequence flanked by an NGG sequence \(Cas9 PAM). This 20 nt sequence will be the gRNA sequence that will be inserted in the BpII site in bRA plasmid. Also, BLASTing the 20nt gRNA sequence to make sure that it is unique is important for specific Cas9 targeting. Add GTTTT to the 3' end of the forward oligo. Take the reverse complement of the 20nt sequence and add GATCA to the 3' end of the reverse oligo \(see the underlined sequences below). When these complementary sequences are duplexed, these additional sequences will serve as overhangs that are complementary to the BpII sites. Oligo1 \(Forward) for the example target sequence: 5'GTTAGCGGTTTGAACGCTGCGTTTT3' Oligo2 \(Reverse) for the example target sequence : 5'GCAGCGTTCAAACCGCTAACGATCA3' b) Resuspend each of the oligos in 1X TE to bring the concentrations to 100uM c) Duplexing the oligos: 5µl Oligo1 \(100uM) 5µl Oligo2 \(100uM) 2µl of ligase buffer 8µl TE 20µl total Heat up the oligos to 100°C, using a water or sand bath or PCR machine for 5 minutes and let it cool down to room temperature on bench. III) Duplexed oligos in the above concentration \(25uM) are more than 1000-fold concentrated than the BpII-digested plasmid \(5ng/µl ~ 1×10^{-15} M). So you have to serially dilute the duplexed oligos more than 1000-fold before setting up the ligation reaction. IV) Ligation: 3µl gel purified vector \(~5ng/µl) 5µl ~1000-fold diluted duplexed insert 1µl 10x ligase buffer 1µl ligase 10µl total \(incubate for 1 hour at room temperature) V) Transformation into bacteria \(standard protocol) Thaw 100 µl competent cells on ice. Add it directly onto 10µl ligation reaction. Incubate on ice for 15mins, heat shock at 42C for 90 seconds, then incubate on ice for 5 minutes. Recover the cells in 500 µl LB at 37 C for 45 minutes and plate onto LB-amp plates. Note: It is a

good idea to set up a control reaction with no gRNA insert to see the frequency of self-ligation. The colonies that pop up on the control plate can also be used as negative control for the colony PCR indicated below. VI) Check the insertion of gRNA into bRA plasmids by colony PCR using the following primers a) Forward gRNA oligo \(5'NNN…NNNGTTTT 3') b) AGCTGAATGAAGCCATACCAAACGA \(Ampicillin reverse primer) The percent of correct colonies is ~50-70%. V) Pick two colonies confirmed by PCR, and extract the plasmids. Send them for sequencing for further confirmation. Universal M13 primer can be used for sequencing. Check for the disruption of BplI cut site and make sure that gRNA sequence is inserted. Integration of mutations to the genome by using Cas9 In our experience, variations of this method can be used to introduce small \(100 bp) or large \(800 bp and probably larger) deletions, insertions, or point mutations. For de novo mutations, 80mers or gblocks \(larger dsDNA oligomers) can be used. But any DNA sequence that has homology to the site that you want to manipulate can serve as a template for Cas9-directed gene manipulation. For example, this template sequence can be amplified from a plasmid and a PCR product can be transformed in the cell together with the Cas9 plasmid. I) Pick a gRNA sequence that is nearby to the sequence that you want to modify. This is the site that Cas9 will bind and cut. Make sure that the sequence is flanked by a PAM sequence \(NGG), has a relatively high GC content and is unique. The PAM could be on either the Watson or Crick strand; facing towards or away from the site you'd like to manipulate. II) Clone the gRNA sequence into the BplI site of bRA89 or bRA90 plasmid as indicated above. The plasmids are essentially the same; but bRA90 is marked with LEU2, and bRA89 is marked with HPH. These plasmids have Cas9 under the control of Pgk1 promoter that is constitutively active. This means, after the plasmid is transformed in the cell, Cas9 will be produced and will continuously cut the targeted sequence. In the absence of a template, most survivors on the transformation plate will have a deletion by NHEJ. If you wish to make small templated modifications of the sequence, you need to make sure that you modify the gRNA or NGG site that Cas9 will bind in the genome together with the sequence you want to alter. III) For introducing deletions, design an single-stranded 80mer template that just consists of 40nts upstream and 40nts downstream of the sequence that you want to delete. In this case, it is more convenient to design the Cas9 gRNA so that it falls within the deletion. Because this way, after the Cas9-directed repair event is complete, gRNA sequence in the genome will be disrupted and Cas9 cannot cut this sequence again. Example sequence: Genomic sequence: nnn-40nts-nnnn—-sequence to be deleted——mmmmm-40nts-mmmm 80mer template: nnn-40nts-nnnn mmmmm-40nts-mmmm You can order the 80mer as a standard oligo, dilute it to 100uM, transform $_{30\mu l \text{ of this together with}}$ 15µl of purified Cas9 plasmid. Plate the cells directly on LEU2 dropout medium for bRA90. For bRA89, plate on YEPD and replica-plate on HPH the next day. Follow the same steps for integrations, and design cassettes that have ~40bps homology on both sides of the sequence you want to integrate. However, in the integration case, you have to mutate the gRNA site in the genome to prevent continuous Cas9 cutting. If the gRNA sequence does not fall into an ORF, the easiest way of doing this is to mutate it to random nucleotides. But keep in mind that not all the codons are used equally, so taking advantage of yeast codon table could be useful. IV) Confirm the genetic manipulation integrated into the genome with Cas9 first by PCR and then sequencing. For large insertions or deletions, you can design PCR reactions that will give products that differ in size depending on the success of Cas9 targeting. For point mutations, you can design oligos that will only anneal to the mutated sequence \(i.e.

falls exactly on the point mutation). You run a gradient PCR to find the best working conditions. You should include a negative control in order to evaluate the results. Also, it is good practice to lose the Cas9 plasmid from the cell. For this, streak the cells onto YEPD and screen for the colonies that are Leu- or HPH- \(depending on the bRA plasmid used). Plasmid retention rate for bRA89/90 is around 70-80%. As a final note; some gRNA sequences fail for unknown reasons \(~10%). Nucleosome positioning and possibly other protein binding at the cleavage site can impair cutting.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

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