CRISPR-Directed Mitotic Recombination Enables Genetic Mapping Without Crosses

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Abstract

Please contact Dr. Steven Wilhelm (wilhelm@utk.edu) for additional information regarding this protocol.

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Guidelines

Mutations and off-target effects
The method has various safeguards against the effects of mutations and off-target cutting. Most importantly, unlike in many uses of CRISPR to create DSBs, the rate of indel formation at the DSB site should be low, as LOH occurs through high fidelity HR, rather than error-prone non-homologous end joining. In the LOH process, the targeted cut site is gene-converted to the PAM-less allele, abolishing further cutting by Cas9. Off-target LOH events are uncovered in our whole-genome genotyping. Finally, in genetic mapping using LOH events a positive signal would typically require multiple different recombination events to generate similar phenotypes, so gRNA-specific off-target effects or mutations should not produce false positive signals.

We checked whether the LOH process introduced mutations at the targeted cut site by Sanger sequencing in 72 independent LOH lines, and did not observe any mutations. We amplified genomic DNA in an interval surrounding the cut sites of 78 individuals, sampled from both the Chr 7L panel and the manganese fine-mapping panel. The PCR products were Sanger sequenced from both ends; we obtained high quality sequence data from both reads for 72 of the 78 individuals. The resulting data were analyzed using the R package SangerseqR (34) to make homozygous as well as heterozygous base calls. Heterozygous calls were made with a cutoff of 0.25 for the ratio of intensity for the secondary basecall relative to the intensity of the primary basecall (default is 0.33). Mutation calls were made if a position had an observed mutation in both of the paired sequencing reads.

Power calculations
We investigated the relationship between statistical power, effect size, and proximity of a QTL to the telomere, in the panel of 384 LOH strains with LOH targeted uniformly across Chr 7L. With this panel design we expect power to detect a QTL to be highest near the telomere and lower for QTL near the centromere. Smaller fractions of lines will be homozygous for polymorphic positions near the

centromere than for polymorphic positions near the telomere. We simulated a single additive QTL at each variant site on Chr 7L using our observed genotype data, by generating a simulated phenotype for each line drawn from a normal distribution with variance 1 and mean 0, β, or 2β, depending on the line's genotype at the variant (homozygous BY, heterozygous BY/RM, or homozygous RM). Simulated phenotypes were generated for the 384 LOH lines while varying the simulated QTL's β from 0.25 to 5, at each of 2000 BY/RM polymorphic positions along Chr 7L, 100 times. Heritability (h2) was calculated as the ratio of var(g) to var(p). QTL mapping was performed as described above. Power was calculated at each QTL position and effect size as the fraction of simulations out of 100 with a LOD greater than 3 at the simulated QTL position.

Data deposition
Code and data can be found at https://github.com/joshsbloom/crispr_loh; sequencing data is deposited at the Sequence Read Archive under accession SRP072527.

Protocol

Preparing Yeast Strains
Step 1.

Start with a diploid yeast strain formed by mating a genetically diverse pair of yeast strains with known genotypes. The diploid yeast strain that is used should be autoptrophic for uracil and leucine.

Preparing Yeast Strains
Step 2.

Select a chromosome arm for targeted LOH. Knock in a TDH3-driven GFP that is amplified from YPW1180 from the Wittkopp lab, heterozygously, at the end of the chromosome, using a standard lithium acetate yeast transformation protocol.

Preparing Yeast Strains
Step 3.

Perform PCR to check which chromosome arm was tagged with GFP in the resulting colonies.

Preparing Yeast Strains
Step 4.

Pick a pair of strains, one with each chromosome tagged.

Preparing Yeast Strains
Step 5.

Transform in p415-GalL-Cas9-CYC1t, also using a standard lithium acetate yeast transformation protocol.

NOTES

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p415GalL-Cas9-CYC1t can be found and purchased at https://www.addgene.org/43804/

Preparing gRNA Plasmids

Step 6.

Select gRNAs target sites evenly spaced around the area of the chromosome arm that you want to create LOH events in: 20 base sequences, followed in the genome by NGG. An important constrain is that each site should only be present on one of the two haplotypes.

Preparing gRNA Plasmids

Step 7.

For each gRNA you wish to generate, design primers of the formgcagtgaaagataaatgatc*NNNNNNNNNNNNNNNNNNN*gttttagagctagaaatagc and ctatagggcgaattgggtac, where the italicized sequence matches the desired targeting sequence, and use them to PCR fragments from p426-SNR52p-gRNA.CAN1.Y-SUP4t.

NOTES

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p426-SNR52p-gRNA.CAN1.Y-SUP4t can be found and purchased at https://www.addgene.org/43803/

Preparing gRNA Plasmids

Step 8.

Miniprep p426-SNr52p-gRNA.CAN1.Y-SUP4t from dam- dcm- bacteria (NEB). Do this step so that the plasmid is free of DNA methylation.

REAGENTS

QIAprep Spin Miniprep Kit 27104 by Qiagen

Preparing gRNA Plasmids

Step 9.

Digest with ClaI and EagI and gel purify.

Preparing gRNA Plasmids

Step 10.

Use a Gibson Assembly protocol to clone the PCR above into the digested plasmid above.

NOTES

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You can find the Gibson Assembly protocol

at https://www.protocols.io/view/Gibson-Assembly-Master-Mix-Assembly-E2611-imsupm

Preparing gRNA Plasmids

Step 11.

Miniprep and sequence to confirm plasmid sequence.

REAGENTS

QIAprep Spin Miniprep Kit 27104 by Qiagen

Preparing gRNA Plasmids

Step 12.

Transform your gRNA plasmids into the yeast strain above.

Plasmid and Strain Construction

Step 13.

Generate gRNA plasmids targeting various sites on Chr 7L that are variable between BY and RM with Gibson Assembly using plasmid p426-SNR52p-gRNA.CAN1.Y-SUP4t provided by George Church.

NOTES

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You can find the specific gRNA targeted sites in a .doc file attached under the "more" section of this protocol.

Plasmid and Strain Construction

Step 14.

Amplify PCR products from p426-SNR52p-gRNA.CAN1.Y-SUP4t with primers that introduce desired 20 bp targeting site sequences. These PCR products are Gibson Assembled into the apprroximately 6000 bp ClaI/KpnI digestion product of p426-SNR52p-gRNA.CAN1.Y-SUP4t. All polymorphisms targeted change an NGG PAM to a sequence other than NGG or NAG.

Plasmid and Strain Construction

Step 15.

The strains used in this study are listed below. The parent strain used for BY was

BY4742 (MATα leu2Δ his3Δ ura3Δ lys2Δ), and for RM was YLK2442 (MATa leu2Δ his3Δ::NatMX ura3Δ ho::HygMX AMN1BY).

NOTES

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Name	Genotype	Notes	Source
YLK2442	MATa leu2Δ his3Δ::NatMX ura3Δ ho::HygMX AMN1_BY	RM parent	Amy Caudy
YLK3218	MATα leu2Δ his3Δ ura3Δ lys2Δ	BY parent	Steven Clarke
YLK3219	YLK2442xYLK3218 HXK2_BY::GFP::HIS ADE6_BY::mCherry::KanMX [p415 GalL-Cas9-Cyc1t]	For Chr 7L LOH, gaining RM	This study
YLK3220	YLK2442xYLK3218 HXK2_RM::GFP::HIS ADE6_RM::mCherry::KanMX [p415 GalL-Cas9-Cyc1t]	For Chr 7L LOH, gaining BY	This study
YLK3221	MATa his3Δ leu2Δ met15Δ ura3Δ nej1Δ::KanMX [p415 GalL-Cas9-Cyc1t]	BY, for variant replacement	This study

Supplementary Table 2: Strains used in this study.

Plasmid and Strain Construction

Step 16.

Use standard lithium acetate procedures for transformations of yeast.

Plasmid and Strain Construction

Step 17.

Facilitate isolation of cells that have undergone LOH, HXK2, which resides 25 kb from the Chr 7L telomere, should be tagged with GFP on either the BY or RM chromosome, by transferring the GFP tag of HXK2 from the GFP-tagged collection into the BY/RM hybrid.

Plasmid and Strain Construction

Step 18.

Place a KanMX selectable marker in *cis* on the appropriate Chr 7R homolog (downstream of ADE6) to allow selection against cells that had lost the GFP through loss of the entire Chr 7.

Plasmid and Strain Construction

Step 19.

Introduce Cas9 on a plasmid (p415-GalL-Cas9-CYClt, also provided by George Church) to produce YLK3219 and YLK3220.

Plasmid and Strain Construction

Step 20.

Transform these strains with the gRNA plasmids whose construction is described above, and grow

overnight in defined media, after which GFP(-) cells are isolated by flow cytometry (FCM) using a Bio-Rad S3e apparatus from Bio-Rad Laboratories, Hercules, CA.

Plasmid and Strain Construction

Step 21.

In this study, approximately 10% of cells with gal-induced Cas9 showed loss of GFP, with some variation dependent on the gRNA used. If this occurs, plate the sorted fraction on YPD+G418, and manually screen the resultant colonies to confirm GFP loss.

Plasmid and Strain Construction

Step 22.

Pick 384GFP(-) colonies (approximately 4 from each gRNA) into 900 µL YPD in 96-well 2 mL deep-well plates, and grown 2 days without shaking at 30°C to stationary phase.

Plasmid and Strain Construction

Step 23.

Freeze 100 µL of saturated culture of each line at -80°C

Plasmid and Strain Construction

Step 24.

Extract DNA from the rest for genotyping, with the EX-96 Tissue DNA Kit from Omega Bio-tek, Norcross, GA.

Sequencing, Genotype Calling

Step 25.

For genotyping the panel targeting all of Chr 7L, construct whole-genome Illumina sequencing libraries as described in Bloom, et al.

NOTES

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J. S. Bloom, I. M. Ehrenreich, W. T. Loo, T.-L. V. Lite, L. Kruglyak, Finding the sources of missing heritability in a yeast cross. Nature 494, 234–237 (2013). Medline doi:10.1038/nature11867

Sequencing, Genotype Calling

Step 26.

Pool libraries from lines into two batches of 192, each of which should be sequenced on a Miseq Sequencer from Illumina, San Diego, CA with PE150 runs.

Sequencing, Genotype Calling

Step 27.

Trim the reads with adapter sequences using trimmomatic 0.32, and align to the reference sacCer3

genome using bwa mem.

Sequencing, Genotype Calling

Step 28.

Use the GATK v 3.4 UnifiedGenotyper to call variants at high-quality variant sites that were previously identified between the parent strains.

Sequencing, Genotype Calling

Step 29.

Using custom R scripts, extract the genotype likelihoods for the homozygous BY, heterozygous, and homozygous RM states from each sample at each known variant site between the parent strains. Filter the low quality sites.

Sequencing, Genotype Calling

Step 30.

Use a hidden Markov model to calculate the posterior probability at each variant site in the genome that a line is homozygous BY, heterozygous, or homozygous RM. Variant sites should be called one of these three states by choosing the state with the highest posterior probability.

Sequencing, Genotype Calling

Step 31.

Genotype the lines from the fine-mapping panel in the same way, except that a single pool of the libraries of 384 lines should be sequenced once on a Miseq Sequencer with PE150 reads and again with an additional PE150 rapid-run flow cell on a Hiseq 4000 Sequencing System by Illumina.

Sequencing, Genotype Calling

Step 32.

Merge BAM files for downstream analysis.

Sequencing, Genotype Calling

Step 33.

Carry out an analysis on 358 lines with genotypes that passed quality control filters.

Sequencing, Genotype Calling

Step 34.

Re-genotype select lines with called recombination events close to the called causal polymorphisms using Sanger sequencing at polymorphisms in the QTL.

NOTES

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In this study, genotyping uncovered lines with both simple recombination events and complex

recombination events featuring a mix of gene conversion and recombination.

Sequencing, Genotype Calling

Step 35.

For determining linkage, reduce the variant site set to only include variant sites at least 300 bp apart from each other. The cM distance between a given pair of variants should be calculated as the percentage of chromosomes with a nonparental genotype. For the LOH panel, linkage should be calculated on haplotype inferred from the diploid genotypes.

Phenotyping

Step 36.

Thaw the 384 frozen lines, array randomly into two 384-well plates, and then phenotype as previou

Phenotyping

Step 37.

Pin the lines briefly from frozen stocks to liquid YPD, grow for two days at 30ºC without shaking, and then pin onto agar plates with a custom pinning robot from S&P Robotics, Toronto, Canada.

Phenotyping

Step 38.

Pictures were taken of colony growth after 48 hrs.

Phenotyping

Step 39.

Measure using functions from the EBImage R Package.

Phenotyping

Step 40.

Perform minimal filtering to remove measurements affected by pinning or contamination artifacts.

Phenotyping

Step 41.

Remove colonies at the edge of the plate if there is a colony with a radii significantly different than the radii of colonies on the rest of the plate.

Phenotyping

Step 42.

Phenotype the two randomized plates of 384 LOH lines, and average colony measurements across the two replicates for downstream analysis. In addition, phenotype the 768 segregant lines from the previous linkage analysis between the same parental strains in parallel, for the same traits, to allow controlled comparison.

NOTES

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Conditions to test: 92 mM calcium chloride, 6 mM copper sulfate, 1.26 mM diamide, 0.25% DMSO, 2% lactate, 91.7 mM lithium chloride, 10 mM manganese sulfate, 2.5 mg/mL neomycin, 0.1% sodium dodecyl sulfate, 2% trehalose, and growth at 15ºC or 30°C on YPD.

Phenotyping

Step 43.

Use the same methods as described above to generate and phenotype the fine-mapping panel targeting a region of Chr 7L, but with all lines derived from 3 gRNAs as shown in supplementary table 1.

QTL Mapping

Step 44.

Detect QTL using an additive linear model with genotypes of homozygous BY, heterozygous BY/RM, and homozygous RM coded as 0, 1, and 2.

QTL Mapping

Step 45.

Convert the R^2 from the model to a LOD score as descrived in Lynch and Walsh 1998.

NOTES

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M. Lynch, B. Walsh, in Genetics and Analysis of Quantitative Traits (Sinauer, 1998), pp. 319– 532.

QTL Mapping

Step 46.

Calculate 5% family-wise error rate significance thresholds per trait using 1000 permutations of phenotypes to strain labels.

QTL Mapping

Step 47.

Map the QTLs in the panel of 768 by/RM segregants as in Bloom et al. Perform all QTL scans on Chr 7L only.

NOTES

Alyssa Alsante 12 May 2017

J. S. Bloom, I. Kotenko, M. J. Sadhu, S. Treusch, F. W. Albert, L. Kruglyak, Genetic interactions contribute less than additive effects to quantitative trait variation in yeast. Nat. Commun. 6, 8712 (2015). Medline doi:10.1038/ncomms9712

Variant Replacements

Step 48.

Do variant replacements using CRISPR to cut near the site targeted for mutation, while providing a repair template carrying the desired mutation embedded in a >100 bp region homologous to the targeted site.

NOTES

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If the desired mutation did not itself disrupt the PAM site used to target Cas9, the repair template was designed to additionally carry a PAM-disrupting synonymous mutation. All such PAM-disruping synonymous mutations were also introduced individually, as controls.

Variant Replacements

Step 49.

Synthesize the DNA fragments carrying the desired gRNA as well as the appropriate repair template as gBlocks (Integrated DNA Technologies, Coralville, IA), and then clone into p426-SNR52p-gRNA.CAN1.Y-SUP4t by Gibson Assembly.

Variant Replacements

Step 50.

Introduce changes in a BY strain with a *nej1Δ* mutation, such that it lacks non-homologous end-joining activity, to bias the repair process towards homologous recombination using the repair template.

Variant Replacements

Step 51.

Transform this strain with the same Cas9-carrying plasmid as above to produce YLK3221.

Variant Replacements

Step 52.

Transform YLK3221 with the plasmids described above, and then grow overnight at 30°C in defined media with galactose as a carbon source to stimulate Cas9 activity.

Variant Replacements

Step 53.

Plate cultures at low density to allow growth of single colonies on glucose media to arrest Cas9 activity.

Variant Replacements

Step 54.

Pick 22 to 32 colonies from each variant replacement experiment into 2 mL deep-well plates and grow and process as above for DNA extraction, with the genotyping done through Sanger sequencing.

Variant Replacements

Step 55.

Lines without mutations uncovered in the genotyping should be used as unmutagenized BY controls; there were 106 total in this study.

Variant Replacements

Step 56.

Phenotype as previously descived, with the two randomized 384-well plates also containing 64 unmutagenized RM parent lines for comparison.

Isolating Cells with LOH

Step 57.

Start a 2 mL 30ºC overnight culture of each gRNA-containing strain in YNB+CSM-LEU-URA+ 2% galactose yeast media. Use a GFP(-) strain as a negative control, and a strain without a gRNA grown in media supplemented to allow its growth, or with an empty URA plasmid as a positive control.

Isolating Cells with LOH

Step 58.

After 24 hrs, dilute to an OD600 of 0.1 in 2 mL YNB+CSM-LEU-URA+ 2% galactose yeast media, and grow 4 hrs at 30ºC.

DURATION

04:00:00

Isolating Cells with LOH

Step 59.

Pellet 1 mL of cells in a tabletop centrifuge for 15 sec at full speed.

DURATION

00:00:15

Isolating Cells with LOH

Step 60.

Resuspend pellets in 1 mL phosphate buffered saline.

REAGENTS

- 1X PBS (Phosphate-buffered saline) by Contributed by users

Isolating Cells with LOH

Step 61.

Check fluorescence of samples via Flow Cytometer. Use the positive and negative conrtols to set the gates on the FCM to isolate GFP(-) cells without isolating GFP(+) cells.

NOTES

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If using the optional RFP, set gates to isolate RFP(+) cells without isolating RFP(-) cells.

Isolating Cells with LOH

Step 62.

Sort the GAL-induced LOH cells by sorting for GFP(-) cells from each gRNA-containing culture.

NOTES

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Alternatively, you can plate the cells at ~200-250 cells/plate. You should be able to identify GFP(-) colonies with any apparatus that visualizes fluorescencce on plates.

Isolating Cells with LOH

Step 63.

Plate 50+ GFP(-) cells from each culture on YNB+CSM-LEU-URA+ 2% glucose plates.

Isolating Cells with LOH

Step 64.

Once they have grown colonies, pick the desired number of colonies, and grow them in 96-well deep-well plates in YNB+CSM-LEU-URA+ 2% glucose at 30ºC for 2 days.

Isolating Cells with LOH

Step 65.

Save 100 uL in 96-well plates at -80ºC with 100 uL of 40% glycerol.

Isolating Cells with LOH

Step 66.

Isolate genomic DNA from the remaining samples with the Qiagen DNeasy 96 Blood & Tissue Kit, with their yeast genomic DNA isolation protocol.

REAGENTS

QIAgen DNeasy Blood and Tissue Kit, 50 rxn 69504 by Qiagen

Genotyping

Step 67.

Use an Illumina Nextera protocol to generate sequencing libraries, with Illumina's 96-plex indexing. Sequence with 150 paired-end sequencing on an Illumina HiSeq.