Video Article

Gene-targeted Random Mutagenesis to Select Heterochromatin-destabilizing Proteasome Mutants in Fission Yeast

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URL: https://www.jove.com/video/57499
DOI: doi:10.3791/57499

Keywords: Genetics, Issue 135, Random mutagenesis, fission yeast, heterochromatin, error-prone PCR, centromere, gene targeting

Date Published: 5/15/2018

Citation: Seo, H.D., Lee, D. Gene-targeted Random Mutagenesis to Select Heterochromatin-destabilizing Proteasome Mutants in Fission Yeast. *J. Vis. Exp.* (135), e57499, doi:10.3791/57499 (2018).

Abstract

Random mutagenesis of a target gene is commonly used to identify mutations that yield the desired phenotype. Of the methods that may be used to achieve random mutagenesis, error-prone PCR is a convenient and efficient strategy for generating a diverse pool of mutants (*i.e.*, a mutant library). Error-prone PCR is the method of choice when a researcher seeks to mutate a pre-defined region, such as the coding region of a gene while leaving other genomic regions unaffected. After the mutant library is amplified by error-prone PCR, it must be cloned into a suitable plasmid. The size of the library generated by error-prone PCR is constrained by the efficiency of the cloning step. However, in the fission yeast, *Schizosaccharomyces pombe*, the cloning step can be replaced by the use of a highly efficient one-step fusion PCR to generate constructs for transformation. Mutants of desired phenotypes may then be selected using appropriate reporters. Here, we describe this strategy in detail, taking as an example, a reporter inserted at centromeric heterochromatin.

Video Link

The video component of this article can be found at https://www.jove.com/video/57499/

Introduction

Forward genetics is a classical method in which researchers seek naturally occurring mutants that display a particular phenotype, and perform genetic analyses. In reverse genetics, mutations are introduced into a gene of interest and the phenotype is examined. In the latter case, random mutagenesis of a target gene is often used to generate a pool of mutants that are subsequently selected for desired phenotypes, such as temperature sensitivity or altered enzymatic activity. Various methods may be used to achieve random mutagenesis, including error-prone PCR[1]; UV irradiation[2]; chemical mutagens, such as ethyl methanesulfonate (EMS) or nitrous acid[3]; the use of temporary mutator strains, such as those over-expressing *mutD5* [4]; and DNA shuffling[5].

Here, we describe a reverse-genetics strategy in which we utilize error-prone PCR to generate mutant pools for a target gene in the fission yeast. As one might guess from its name, this method generates mutations by deliberately introducing errors during PCR. Unlike other mutagenesis methods, error-prone PCR allows the user to define the region to be mutagenized. This makes it particularly useful in efforts to study the function of a protein/domain of interest.

To demonstrate this random mutagenesis procedure, we herein use *rpt4+*, which encodes the 19S proteasome subunit, as an example. Rpt4 has been shown to have proteolysis-independent functions in organisms other than fission yeast[6,7,8,9], and a defect in proteolysis could cause indirect effects by altering proteins levels. We, therefore, screened for mutants that triggered proteolysis-independent changes, with the goal of investigating the function of the proteasome on heterochromatin.

Error-prone PCR can be applied to any gene region by tuning the location at which the primers bind. Mutants that exhibit the desired phenotypic change can be identified with appropriate reporters. Here, we utilized an *ade6+* reporter inserted at the centromere 1 outer repeat (*otr*) region[10]. Constitutive heterochromatin is formed at this region[11], so the *ade6+* reporter is silenced in the wild-type condition; this is indicated by red colonies[10]. A mutation that destabilizes the constitutive heterochromatin at the centromere will lead to the expression of the *ade6+* reporter, which is visualized as white colonies.

Protocol

1. Preparation of Media

1. Prepare Yeast Extract with supplements (YES), YES without adenine (YES Low Ade), Pombe Glutamate medium (PMG[12]), and PMG without adenine (PMG-Ade) by mixing the components as described in **Table 1**. YES-Ade (Low Ade) and PMG-Ade plates have all of the same components, but the adenine is omitted from the latter.

1. Use the following salt (50x) stock: 52.5 g/L $MgCl_2 \cdot 6H_2O$, 2 g/L $CaCl_2 \cdot 2H_2O$, 50 g/L KCl, 2 g/L Na_2SO_4 in distilled water. Filter sterilize using a 0.22-µm pore-size filter and store at 4 °C.
2. Use the following vitamin (1,000x) stock: 1 g/L pantothenate, 10 g/L nicotinic acid, 10 g/L inositol, 10 mg/L biotin in distilled water. Filter sterilize using a 0.22-µm pore-size filter and store at 4 °C.
3. Use the following mineral (10,000x) stock: 5 g/L boric acid, 4 g/L $MnSO_4$, 4 g/L $ZnSO_4 \cdot 7H_2O$, 2 g/L $FeCl_2 \cdot 4H_2O$, 0.4 g/L MoO_3, 1 g/L KI, 0.4 g/L $CuSO_4 \cdot 5H_2O$, 10 g/L citric acid in distilled water. Filter sterilize using a 0.22-µm pore-size filter and store at 4 °C.
NOTE: YES liquid medium can be made by omitting the agar.

2. Autoclave the medium and cool it to below 60 °C while stirring with a stir bar, at the rate of 200 rpm.
3. For YES+G418 (geneticin) plates, add 1 mL/L G418 stock solution to the YES medium.
1. Use the following G418 (1,000x) stock: 100 mg/mL G418 in distilled water. Filter sterilize using a 0.22-µm pore-size filter, aliquot to 1.5 mL centrifuge tubes, and store at -20 °C.

4. Stir for another 5 - 10 min and aliquot the media into 90-mm Petri dishes (1 L of medium aliquots to roughly 40 Petri dishes. Store the plates at 4 °C.

2. Cloning of *rpt4+* and its 5'/3' UTRs

1. Perform PCR with primers p1 and p2 (**Figure 1A**) using fission yeast genomic DNA (gDNA) as the template in a total volume of 50 µL (~1 µg DNA, 20 U enzyme, 1x reaction buffer), and applying the reaction cycles described in **Table 2 to** amplify a 3,315-base pair (bp) fragment comprising *rpt4+* and its 5'/3' UTRs. Purify the PCR product using a purification kit[13] as directed by the manufacturer (**Figure 1A**).
2. Digest the pBlueScript KS(-) vector[14] and the amplified DNA fragment containing *rpt4+* with its 5'/3' UTRs with *BamH1* and *Xho1* in a total volume of 20 µL (~1 µg DNA, 20 U enzyme, 1x digestion buffer) at 37 °C in a water bath for 16 - 18 h (**Figure 1B**).
3. Gel purify the digested vector (1.2% agarose gel) and DNA insert by performing TAE-based agarose gel electrophoresis (100 V, 1 h), excising the DNA bands of the desired sizes, and isolating the DNA with a gel extraction kit[15].
4. Ligate the vector and DNA insert using T4 DNA ligase by performing a 20-µL ligation reaction containing 50 - 100 ng of the vector DNA, a ~3-fold molar excess of the insert DNA, 400 U T4 DNA ligase, and 1x ligation buffer. Incubate this mixture at 18 °C for 16 - 18 h.
5. Transform the ligated DNA into 100 µL of *E. coli* DH5α by applying heat shock for 70 s at 42 °C. Add 1 mL of LB and incubate for 1 h at 37 °C for recovery. Perform centrifugation (13,800 x g), discard the supernatant, plate all of the transformed *E. coli* cells on LB-Ampicillin (LA) plates, and incubate at 37 °C for 16 h.
6. Pick 4 - 8 colonies with toothpicks and grow overnight at 37 °C in 3 mL LA medium.
7. Isolate the plasmids with a plasmid purification kit[16] used according to the manufacturer's protocol and perform analytical restriction enzyme digestions with 5 µL of the isolated plasmid, 5 U of each restriction enzyme (*BamH1* and *Xho1*) and 1x restriction buffer. Incubate the reaction mixture at 37 °C in a water bath for 3 h. Confirm the cloning by sequencing candidate plasmids.

3. Introduction of the Silent Mutation (*Xho1* Restriction Site)

1. Design a pair of 33-bp primers (p3 and p4) that contain the desired mutation in the otherwise complementary sequence.
2. Perform PCR with high-fidelity polymerase[17] (**Figure 1C**) in a total volume of 25 µL (10 ng of cloned plasmid, 2 µL of 10 pmol primer mix, 2 U enzyme, 1x reaction buffer) using the cycling parameters listed in **Table 3**.
3. Digest the template plasmid with *Dpn*I in a total volume of 20 µL (20 U enzyme, 1x digestion buffer) at 37 °C in a water bath for 1 h.
4. Transform, propagate, isolate, and sequence the plasmid containing the silent mutation, as described in steps 2.5 - 2.7.
Note: The silent mutation can also be introduced using a cloning method which allows for the joining of multiple DNA fragments in a single reaction[18].

4. Random Mutagenesis of *rpt4+* by Error-prone PCR

1. Perform error-prone PCR, using the plasmid with the silent mutation (*Xho1* site) as the template, primers p5 and p6, a total volume of 50 µL (the amount of template defined in step 4.1.1, 2 µL of 10 pmol primer mix, 2.5 U enzyme, 1x reaction buffer), and a special polymerase that is designed to generate a high error rate[19] (**Figure 1D**). Perform PCR as described in **Table 2**.
1. Use 4 - 5 µg of template plasmid to control the mutation frequency to 1 bp/kb (kilobase).
Note: A high-concentration (>500 ng/µL) of plasmid, which may be obtained using a Mini-prep kit[20], is required.

2. Use gel electrophoresis to confirm a PCR product of 2670 bp (1.2% agarose gel). Gel purify this PCR product as described in step 2.5.

5. Preparation of Fusion PCR Fragments (KAN, 3'UTR)

1. Perform PCR using pFA6a-KANMX6[21] as the template, primers p7 and p8, and the conditions listed in Table 4 to obtain the marker (KAN) fragment. Gel purify the resulting 1,480-bp fragment as described in step 2.5 (**Figure 1E**).
1. Check if the stop codon of the gene targeted for mutation and the coding region of its adjacent gene are separated by more than 500 bp. The endogenous terminator may not be used when this region is less than 500 bp; rather, the *ADH* terminator should be used instead of the endogenous terminator. The protocol changes required to use the *ADH* terminator are presented in **Table 5**.
Note: These changes only apply when the *ADH* terminator, which is available in the pFA6a-3HA-KANMX6 plasmid, is used instead of the endogenous terminator.

2. Perform PCR with the cloned *rpt4+*-containing vector as the template and primers p9 and p10 in a total volume of 50 µL (20 ng of the cloned plasmid, 2 µL of 10 pmol primer mix, 2 U enzyme, 1x reaction buffer), using the conditions presented in **Table 6**. Gel purify the obtained 506-bp 3'UTR fragment (**Figure 1E**).

6. Generation of the Transformation Construct by Fusion PCR (Figure 1E)

1. Prepare the 3 fragments (mutagenized *rpt4+*, KAN, and the 3'UTR) at 50 ng/µL.
2. Perform fusion PCR of the three fragments using primers p11 and p12, as described in Table 7. (The protocol for fusion PCR is a modification of the original protocol[22].) Purify the major 4,398-bp PCR product.
    1. Use *rpt4+*:KAN:3'UTR fragments at a ratio of 1:3:1 for optimal results.
    Note: The total amount of the insert DNA should not exceed 500 ng. The recommended amount of *rpt4+*:KAN:3'UTR is 50 ng:150 ng:50 ng. The mutagenized region is approximately 1.6 kb, so the minimum number of yeast colonies that would account for all possible combinations of each base being mutated to the other three is 1,600 x 3 = 4,800 colonies. Because one transformation reaction typically yields 400 - 500 colonies, at least 10 reactions worth of fusion PCR construct is needed. This need can be met by simply increasing the reaction amount (*i.e.,* the number of PCR tubes) by a factor of ten.

7. Transformation of Fission Yeast by Electroporation (Figure 1F)

1. Inoculate yeast to 10 mL of YES medium to saturation by incubating it for more than 16 h.
2. Dilute the cells to an optical density at 600 nm (OD_{600}) of 0.2 in 200 mL of YES medium and incubate at 30 °C with shaking for 5 - 6 h, to OD_{600} = 0.6 - 0.8.
3. Concentrate cells to OD_{600} = 30, dispense to four 50-mL conical tubes and chill on ice for 10 min.
4. Harvest the cells by performing centrifugation at 1,050 x g for 3 min at 4 °C. Place the tubes and 10 electro-cuvettes on ice. Perform the steps 7.3 - 7.8 on ice.
5. Discard the supernatant and add 15 mL of 1.2 M sorbitol. Gently shake the tubes to resuspend the cells.
6. Centrifuge the cells at 1050 x g for 3 min at 4 °C.
7. Repeat steps 7.4 and 7.5. Discard the supernatant. Add 500 µL of 1.2 M sorbitol to each tube, resuspend the cells, and collect all of the cells in one 15-ml conical tube.
8. Add 1.2 M sorbitol up to 2.4 mL (OD_{600} = 10 per 0.2 mL). Keep the tube on ice.
9. Add 200 µL of sorbitol-suspended cells (make sure they are fully suspended) to an EP tube containing the fusion PCR construct, mix well, and transfer the sample to an electro-cuvette.
10. Electroporate the cells with the following options: Fungi, ShS (2.00kV, 1 pulse).
11. Add 600 µL of 1.2 M sorbitol to each electro-cuvette for a total volume of 800 µL, and then spread the cells on four YES plates (200 µL/plate). Ten electro-cuvettes will dispense to 40 YES plates.
12. Incubate the plates at 30 °C for 24 h.
13. Perform replica plating to YES+G418 and incubate the plates at 30 °C for 3 days.

8. Selection of Heterochromatin-destabilizing Mutants and Checking for False Positives

1. For each YES+G418 plate, perform replica plating to YES-Ade (Low Ade) and PMG-Ade (No Ade) plates. Incubate the replica plates for 1 - 2 days at 30 °C, until some of the colonies on the YES-Ade plates show a red coloration (**Figure 1G**).
2. Compare YES-Ade and PMG-Ade plates and select cells that show pink or white on the YES-Ade plate and also grow on the PMG-Ade plate. Do not select colonies without growth on PMG-Ade, as they are false positives (*e.g.,* reflecting that the KAN cassette has been integrated at a non-target site elsewhere in the genome).
3. Pick approximately 1 x 10^5 cells from each colony and incubate in 10 µL of SPZ solution containing 2.5 mg/mL zymolyase 100 T at 37 °C for at least 30 min. Use 1 µL of this solution to perform as the starting template for colony PCR (**Figure 1H**).
    1. Make SPZ (50 mL) by mixing 30 mL of 2 M sorbitol, 4.05 mL of 1 M Na_2HPO_4, 0.95 mL NaH_2PO_4 and 15 mL of distilled water (final pH, 7.5). Samples (5 mL) can be supplemented with zymolyase and stored in 500-µL aliquots at -20 °C until use.
4. Perform gel electrophoresis (1.2% agarose gel, 180V, 20 min) to visualize the results of +the colony PCR. Select reactions that exhibit PCR bands of the proper size and digest 5 µL of each reaction product with *Xho*I (4 U enzyme, 1x digestion buffer, 37 °C water bath, 1 h) to screen out false positives (**Figure 1H**).
5. Perform gel electrophoresis to visualize the *XhoI* digests (1.2% Agarose gel, 180 V, 20 min). Mark the colonies whose PCR products are cut by *XhoI*, and patch them to YES+G418 plates (**Figure 1I**).
6. Isolate gDNA from the fission yeast cells and perform sequencing of the PCR products[23]. Compare the obtained sequence with the wild-type sequence to identify mutations (**Figure 1I**).
7. Directly re-introduce the mutation(s) to wild-type cells by transforming them with fusion constructs amplified by PCR from mutant cells[23]. Use spotting to confirm the phenotype in the newly made mutant cells (**Figure 1J**).
    1. Fusion transformation construct can be easily amplified by PCR with primers p1 and p10 using the genomic DNA of mutants as the template in a total volume of 50 µL (~1 µg DNA, 20 U enzyme, 1x reaction buffer), and applying the reaction cycles described in **Table 2**. Transformation of the construct can be done by following the steps 7.1 - 7.13.

Representative Results

The acquired Rpt4 mutants by following the procedures described in **Figure 1** can be analyzed by assessing the colors of the colonies. The colors of the colonies are spotted onto the relevant plates in decreasing cell number in **Figure 2**. The *ade6+* reporter inserted at the

heterochromatin region is silenced in wild-type and shows red colonies in YES-Ade plate. Once the heterochromatin is destabilized and the *ade6+* reporter is expressed, white colonies can be observed in YES-Ade plate as in *clr4Δ* mutant. The screened Rpt4 mutants are as shown. *rpt4-1* mutant shows the most severe heterochromatin destabilization.

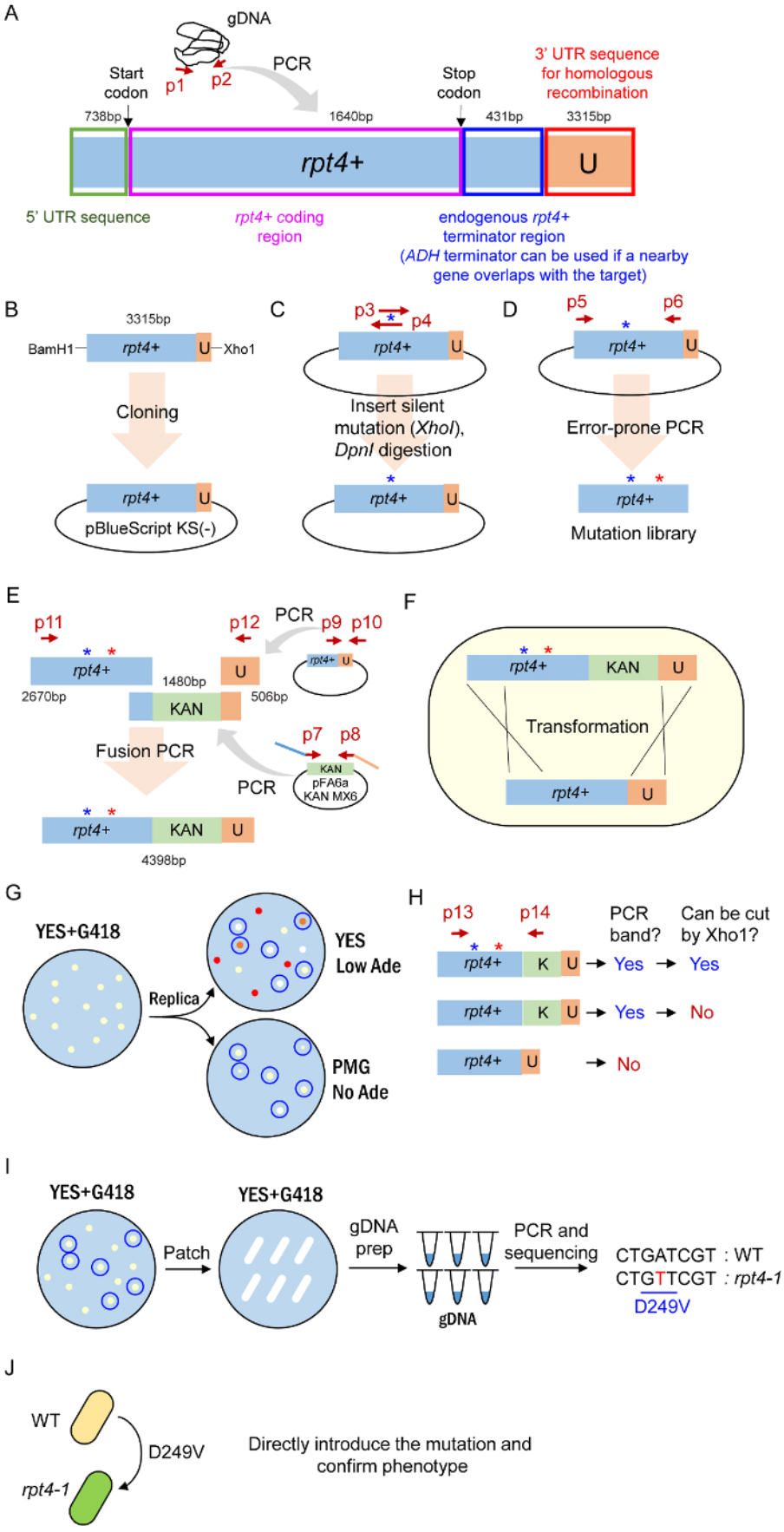
A
gDNA
PCR
Start codon
p1
p2
Stop codon
3' UTR sequence for homologous recombination
738bp
1640bp
431bp
3315bp
rpt4+
U
5' UTR sequence
rpt4+ coding region
endogenous rpt4+ terminator region
(ADH terminator can be used if a nearby gene overlaps with the target)
B
3315bp
BamH1
rpt4+
U
Xho1
Cloning
pBlueScript KS(-)
C
p3
p4
Insert silent mutation (XhoI), DpnI digestion
D
p5
p6
Error-prone PCR
Mutation library
E
p11
p12
PCR
p9
p10
1480bp
2670bp
506bp
KAN
Fusion PCR
p7
p8
pFA6a KAN MX6
4398bp
F
Transformation
G
YES+G418
Replica
YES Low Ade
PMG No Ade
H
p13
p14
PCR band?
Can be cut by Xho1?
Yes
No
I
YES+G418
Patch
gDNA prep
gDNA
PCR and sequencing
CTGATCGT : WT
CTGTTCGT : rpt4-1
D249V
J
WT
D249V
rpt4-1
Directly introduce the mutation and confirm phenotype

Figure 1: Schematic representation of the protocol. (A) Schematic representation of the PCR product obtained by the first round of PCR, which is performed with primers 1 and 2. **(B)** Restriction-based cloning of the *rpt4+* fragment. **(C)** Site-directed mutagenesis of the cloned vector is used to introduce a silent mutation that adds an *XhoI* restriction site. **(D)** Random mutagenesis of the *rpt4+* coding region is performed using error-prone PCR. **(E)** The mutated *rpt4+* fragment, the KAN fragment, and the 3'UTR fragment are joined by fusion PCR to generate a transformation-ready cassette. **(F)** Fission yeast cells are transformed with the fusion PCR construct, which replaces the endogenous *rpt4+* sequence by homologous recombination. **(G)** KAN-selected colonies are replica plated to YES-Ade (Low Ade) and PMG-Ade (No Ade) plates, and positive colonies are selected. **(H)** PCR and subsequent *XhoI* restriction of the PCR product are used to avoid false positives. **(I)** The mutation that causes the phenotype is identified by patching of the selected colonies, propagation of cells, extraction of genomic DNA (gDNA), and sequencing of the relevant portion of *rpt4+*. **(J)** Causative mutations are confirmed (and false positives are ruled out) by directly introducing the mutation to wild-type cells. Please click here to view a larger version of this figure.

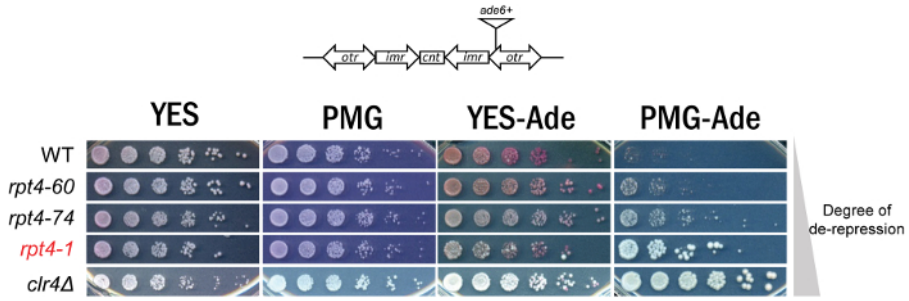

Figure 2: Heterochromatin de-repression mutants of Rpt4. Schematic diagram of the *otr1::ade6+* reporter (top). 5-fold serial dilutions of select screened Rpt4 mutants were spotted onto the indicated plates in order of increasing level of heterochromatin destabilization (bottom). This figure was modified from the article 'The 19S proteasome is directly involved in the regulation of heterochromatin spreading in fission yeast' by Seo *et al.,* 2017[24]. The non-cropped figure can be found in the original article. Please click here to view a larger version of this figure.

Component	Type of plate	
	YES	PMG
Yeast extract	5 g/L	
Glucose	30 g/L	20 g/L
Adenine	0.15 g/L	0.1g/L
Histidine	0.15 g/L	0.1g/L
Leucine	0.15 g/L	0.1g/L
Uracil	0.15 g/L	0.1g/L
Potassium hydrogen pthalate		3 g/L
Na_2HPO_4		2.2 g/L
L-glutamic acid		3.75 g/L
Salts		20 ml/L
Vitamins		1 ml/L
Minerals		0.1 ml/L
Agar	16 g/L	16 g/L

Table 1. Components of YES and PMG plates

Temperature	Time	Cycle(s)
95°C	2 min	1 cycle
95°C	20 s	30 cycles
55°C	30 s	
72°C	2 min	
72°C	8 min	1 cycle
8°C	Hold	

Table 2. Recommended PCR program for site-directed mutagenesis

Temperature	Time	Cycle(s)
95°C	2 min	1 cycle
95°C	30 s	19 cycles
55°C	1 min	
72°C	7 min	
72°C	10 min	1 cycle
8°C	Hold	

Table 3. Recommended PCR program for error-prone PCR

Temperature	Time	Cycle(s)
95°C	2 min	1 cycle
95°C	20 s	30 cycles
55°C	30 s	
72°C	2 min	
72°C	8 min	1 cycle
8°C	Hold	

Table 4. Recommended PCR program for obtaining the KAN fragment

Change	To	Example case of rpt4+
Template vector	pFA6a-3HA-KANMX6	
Vector-binding sequence of p7	ATTACGCTGCTCAGTGCTGA	ttgctgacctgaagaaacttgaaggtacaattgattaccaaaagctttag ATTACGCTGCTCAGTGCTGA
Hanging sequence of p7	The last 50bp sequence including the stop codon	
Hanging sequence of p8	The first 50bp sequence right after the stop codon (complementary sequence)	AATCTTCATCGGTAAACTTATCATTTCATGGCTTTTTGGATATATGTGCA GAATTCGAGCTCGTTTAAAC
Sequence of p9	Start right after the stop codon	tgcacatatatccaaaaagccatgaa
Sequence of p10	Produce ~500bp fragment with p9 (complementary seqeucne)	TAGACGTTTTTCCTCGTTTCTTTGTC

Table 5. Changes needed when the ADH terminator is used instead of the endogenous terminator

Temperature	Time	Cycle(s)
95°C	2 min	1 cycle
95°C	20 s	30 cycles
55°C	30 s	
72°C	1 min	
72°C	8 min	1 cycle
8°C	Hold	

Table 6. Recommended PCR program for obtaining the 3'UTR fragment

Temperature	Time	Ramp	Cycle(s)
94°C	2 min		1 cycle
94°C	20 s		10 cycles
70°C	1 s		
55°C	30 s	0.1 °C/s	
68°C	2:30 min	0.2 °C/s	
94°C	20 s		5 cycles
70°C	1 s		
55°C	30 s	0.1 °C/s	
68°C	2:30 min	0.2 °C/s + 5 s/cycle	
94°C	20 s		10 cycles
70°C	1 s		
55°C	30 s	0.1 °C/s	
68°C	2:30 min	0.2 °C/s + 20 s/cycle	
72°C	10 min		1 cycle
8°C	Hold		

Table 7. Recommended PCR program for fusion PCR

CBL1877	h+	ade6-210 leu1-32 ura4-D18 otr1R (dg-glu) Sph1:ade6

Table S1: Strain used in this study

Primer number	Sequence	Comments
p1	ggg GGATCC tattgagtttcgtttattcgg	Red-colored bases indicate restriction sites
p2	ggg CTCGAG GTACATGTCTTTACAAAGGG	
p3	AAA CTC TTT TAG CTC GAG CCG TGG CGG CAT CTT	Red-colored bases indicate point-mutation sites
p4	AAG ATG CCG CCA CGG CTC GAG CTA AAA GAG TTT	
p5	gacacaagtggtcaattgaattgga	
p6	TTCGTTCAATATATTTAGTTCGTTCTTTACACTT	
p7	ctaaccaaatgcactgaagtgtaaagaacgaactaaatatattgaacgaa CAGCTGAAGCTTCGTACGC	Blue- and orange-colored bases indicate hanging sequence, and black bases indicate the vector-binding sequence
p8	CAAACTATCCTTCCTGGGAAGAGAAGAATTTTTGCATTTTTGAACCGGGT GAATTCGAGCTCGTTTAAAC	
p9	acccggttcaaaaatgc	
p10	GTACATGTCTTTACAAAGGG	
p11	cttaagagaccaagttttgg	
p12	TTTTTCAATGACTCTATCTGG	
p13	gtttcatagcaaatcgttgg	
p14	CTGCAGCGAGGAGCCGTAAT	

Table S2: List of primers used in this study

Discussion

Random mutagenesis via error-prone PCR is a powerful tool for generating a diverse pool of mutants in a given region. This technique is especially useful for studies that seek to assess the function of a protein under a specific circumstance. For example, we herein used error-prone PCR to assess the function of the 19S proteasome subunit, Rpt4, in heterochromatin maintenance. By varying the region targeted by the error-prone PCR and adjusting the screening conditions, we were able to mutate cells at the genomic region of interest and screen for the desired phenotype. Recently, a similar method was used to isolate fission yeast cells harboring mutations in the spindle pole body component[25].

The key advantage of random mutagenesis is that it may be applied to both non-essential and essential genes. Random mutagenesis of essential genes may yield diverse mutants, such as those with subtle and/or partial functional defects that allow cell viability to be sustained, or mutants whose functions are affected only under a specific condition. An example of the latter is a temperature-sensitive mutant. Such mutants may be isolated using 5 mg/L phloxine B, which stains dying cells in red and enables slow-growing cells to be distinguished from dying cells[26].

The critical step of this protocol is the error-prone PCR step. At this step, it is important to control the mutation frequency, which can vary widely depending on the number of PCR cycles and the initial amount of template. We recommend that the user fix the number of PCR cycles and vary

the initial amount of template, as we found this to be a more convenient strategy for optimization. We note that the technique of error-prone PCR has been widely available for decades. If the user chooses, they may seek a method for manually performing error-prone PCR[27] without using a commercially available mutagenesis kit.

As a caution, the false positive rate of this protocol is rather high. Thus, mutant candidates should undergo a series of screenings intended to weed out the false positives. We found that the silent incorporation of a restriction site in the target gene can help avoid the need to subject false positives to the relatively laborious step of sequencing. This is cost-effective, as the mutant candidates may number in the hundreds or even beyond.

Disclosures

The authors have nothing to disclose.

Acknowledgements

Funding support for this project was provided by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Science and ICT (2016R1A2B2006354).

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