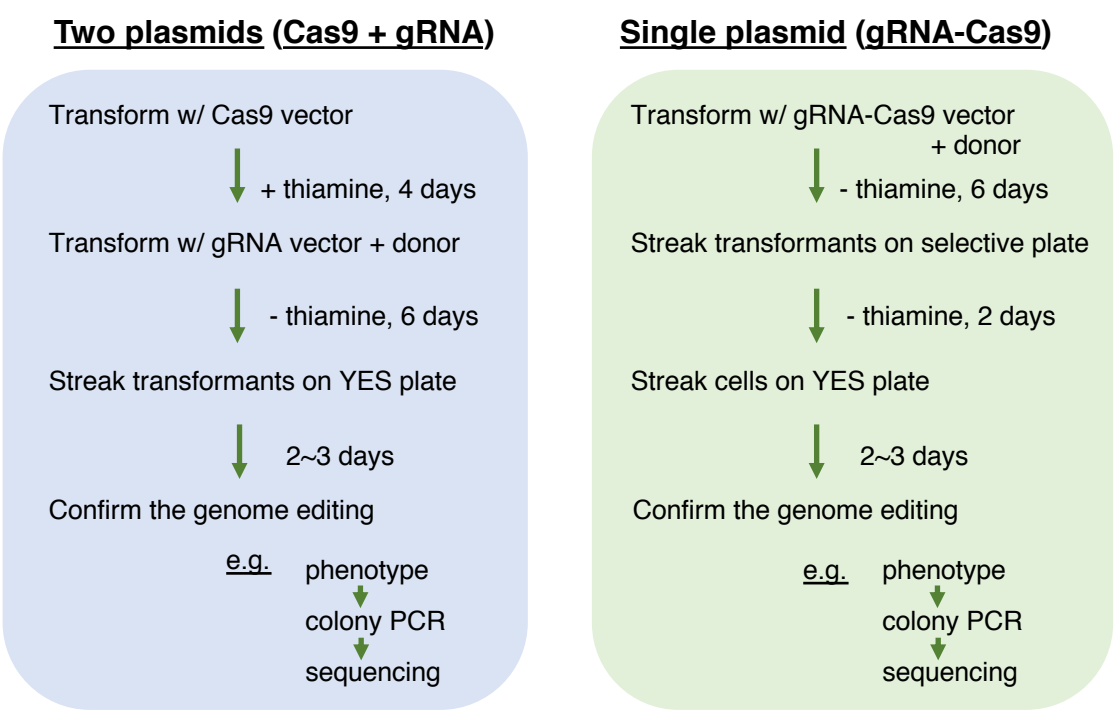
Feb. 26, 2019

Short-homology-mediated CRISPR/Cas9-based method in fission yeast

Hayashi & Tanaka 2019, *G3* https://doi.org/10.1534/g3.118.200976



1. Design gRNA oligomers

1-1. Design gRNAs

Choose gRNA sequence near the position you would like to introduce mutation/insertion. Or, go to the following web sites (e.g. CRISPRdirect) to find the appropriate gRNA candidates. You can input ~500 bp genomic sequence, select the genome of organisms (e.g. fission yeast) and initiate a search. Avoid gRNA that has low GC content (less than 40%).

1-2. Check the gRNA specificity

Choose gRNA that has low number of similar sequences in the genome. Avoid using gRNA that has many A/T sequences near PAM.

1-3. order oligomers for gRNA cloning

gRNA - Fw	`5'-caccXXXXXXXXXXXXXXXXXXXX-3'`
gRNA - Rv	`5'-aaacXXXXXXXXXXXXXXXXXXXX-3'`

Do not include PAM (NGG)!

Web tool for gRNA design

CRISPRdirect	https://crispr.dbcls.jp
E-CRISP	http://www.e-crisp.org/E-CRISP/designcrispr.html
Bähler lab genome Regulation	http://bahlerweb.cs.ucl.ac.uk/cgi-bin/crispr4p/webapp.py

2. Cloning gRNA

2-1. annealing of oligos

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CACCXXXXXXXXXXXXXXXXXX
    XXXXXXXXXXXXXXXXXXCAAA
```

*10 x annealing buffer	3 ul	
gRNA Fw (100 uM)	6 ul	
gRNA Rv (100 uM)	6 ul	
dH_2O	15 ul	/30 ul

Mix the oligos in a PCR tube. Heat at 95˚C 2 min in thermal cycler then leave at R.T for 30 min~1h.
Or you can use a program for annealing (95˚C 2 min, cool-down to 25˚C (- 2˚C/ min) of thermal cycler.

*10 x annealing buffer: 0.1 M Tris pH7.5~8, 0.5 M NaCl, 10 mM EDTA

2-2. digestion gRNA expression plasmid w/ *Bbs*I

***Bbs*I**

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5′..GAAGACNN↓      ..3′
3′..CTTCTGNN(N)4↑  ..5′
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After plasmid DNA preparation, heat for 30 min at 65˚C and keep in a freezer.
Cas9 plasmid is less stable, it is desirable to keep at -80˚C.

Digestion

Incubation at 37˚C, 1 h

10 x buffer	10 ul	
Plasmid (1 ~ug)	X ul	
BbsI (NEB) (10 U/ ul)	0.4 ul	
dH_2O	X ul	/100 ul

Check linearization of plasmid by gel electrophoresis
Heat-inactivation of *Bbs*I (65˚C, 20 min)
EtOH precipitation
(If works, you can purify using column DNA purification kit. Sometimes you might get a low recovery because of large-sized plasmid.)

Keep at -80˚C.

2-3. ligation of annealed oligo and gRNA expression plasmid

Incubation at 16˚C, 30 min

e.g. (TOYOBO Ligation high ver.2)

Ligation high (1/2 volume of DNA)	1 ul	
*Bbs*I digested Plasmid (~10 ng/ul)	1 ul	
1/100 diluted annealed oligo	1 ul	/ 3 ul

2-4. transformation

Add ligated plasmid (~5 ul) to 50 ul of competent cells (e.g. *DH5α*)
Incubate cells on ice, for 30 min
Heat at 42˚C, 30 sec
Cool down on ice, for 2 min
Spread cells on LB-Amp plate
Incubate overnight at 37˚C

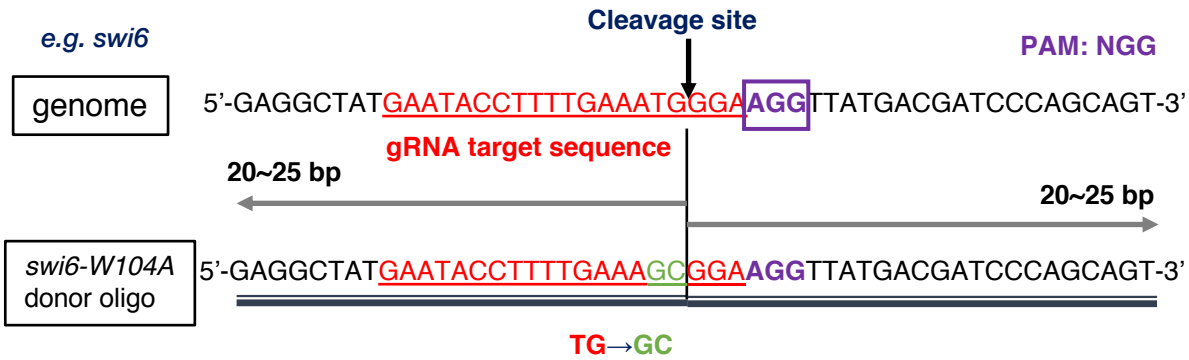
2.5. gRNA insertion check

Grow single colony in LB-Amp broth at 37˚C overnight and isolate plasmid DNA next day using column DNA preparation kit. Heat plasmid DNA at 65˚C for 30 min and keep in a freezer.

For check gRNA insertion, do colony PCR with gRNA Fw and the proper Rv primer if you like. And/or gRNA insertion is confirmed by sequencing.

3. Design donor oligomers

3. 1. for introduced point mutation



Find the cleavage site that is located at - 3 bp from PAM. Choose 40~50 bp of sequence near the cleavage site (20~25 bp of homologous sequences from the cleavage site). The knock-in frequency is very high if the mutation site is located near the cleavage site.

Order donor oligomer (~50 bp). If you would like to introduce mutation at a high frequency, use mixed (Fw and Rv oligomer) or double stranded oligomer for transformation.

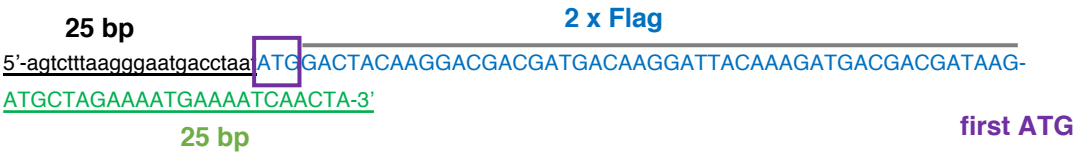
As donor oligos, using single stranded oligo (Fw) is able to introduce genome editing. Mixed and/or double stranded oligo are precisely introduced the knock-in at high frequency.

3. 2. for knock-in tag

Mixed or double stranded oligo are better for the introduction of precise insertion.

3. 2. for knock-in tag (continued)

Order oligomers (~25 bp homology + first ATG + tag + ~25 bp homology).



4. Transformation w/ CRISPR/Cas9 plasmid and donor oligo

- two plasmids protocol -

You can stock the yeast cells carrying Cas9 expression plasmid at -80˚C freezer. For stock, the cells carrying Cas9 expression plasmid are cultured in EMM – uracil + thiamine medium to repress Cas9 expression. Before transformation, the Cas9 expressing cells are cultured in EMM – uracil – thiamine medium for overnight.

- single plasmid protocol -

The yeast cells are cultured in EMM5 – thiamine medium for overnight.

Transformation: e.g. *S.pombe* transformation kit (Wako) (Li-Ac method)

Log phase culture (1~2 x 10^7 cells/ml) is harvested and suspended with the medium to adjust cell number to 1~2 x 10^9 cells/ml.

e.g. harvest 10 ml of 1~2 x 10^7 cells/ml culture and suspend w/ 100~200 ul of medium.

Mixed:

~1 ug of gRNA expression plasmid (or gRNA-Cas9 expression plasmid)
donor DNA (single stranded oligo: 1 nmol, double stranded oligos: 900 pmol)
carrier DNA (e.g. 2 ul of 2 mg/ml salmon testes DNA)
1~2 x 10^7 cells (10 ul)
Transformation reagent (including PEG)(45 ul)

Incubate the mixed cells at 37˚C for 2~3 h. If host cell is temperature-sensitive, you can incubate the cells at permissive temperature (e.g. for 6h~overnight at 25˚C).

Spread the cells gently on selective medium plate

Incubate plate at 32˚C for 6~7 days. If the cell would be obtained temperature sensitive phenotype by genome editing, incubate at permissive temperature (e.g. 10~ days at 25˚C).

5. Confirmation of mutation/insertion introduction

5.1. choose small transformants

do not choose big colonies!

- two plasmids protocol -

Choose small transformants and streak transformants on YES plate to get single colonies. Incubate plate for 2~3 days.

- single plasmid protocol -

Choose small transformants and streak transformants on selective medium plate and incubate plate for 2~3 days. Then, streak the cells on YES plate to get single colonies.

5.2. phenotype check

If your mutant has some phenotypes, you can select the mutants before you confirm the genome editing by sequencing.

5.3. plasmid loss check

Streak single colonies on both of YES and selective medium plate to check the loss of plasmids from the cells. If you use *ura4*$^{+}$ -marked plasmid, you can use 5-FOA plate to remove the plasmids.

5.4. direct colony PCR

Choose colonies that lost the plasmids for direct colony PCR.

e.g.				
	10 x PCR Buffer		1 ul	
	2 mM dNTPs		0.8 ul	
	primers (2.5 uM)	Fw & Rv	0.4 ul each	
	Taq polymerase (5 U/ul)		0.05 ul	
	dH_20		X ul	
	*cells			/10 ul

*touch fresh colony with 200 ul tip and mix well w/ reaction mixture in a PCR tube

94˚C 2 min - 94˚C 30 sec. - 52 ˚C 40 sec.- 72˚C (1 kb/ 1 min) x 36 cycles - 72˚C 10 min - hold 12 ˚C

5.5. Electrophoresis of PCR product

Electrophoresis of PCR products (1 ul) to check the size/ rearrangement/ insertion.

5.6. sequencing

Remove primers and dNTPs from PCR product. You can purify PCR product DNA using purification kit. Or, use ExoSAP (ThermoFisher).

e.g.			
	PCR product	2.5 ul	
	ExoSAP-IT *express*	0.25 ul	
	dH_20	0.75 ul	/1 sample

37˚C 4 min - 80˚C 1 min - hold 4˚C

Use 1 ul of treated PCR product for sequencing analysis.