gRNA design and cloning for the rrk1 fission yeast CRISPR/Cas9 vectors.

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1. **Targeting sequence design**

Near or at the desired mutation site, choose a target with a PAM (red)

```
5'NNNNNNNNNNNNNNNNNNNNNNGGNNN3'
3'NNNNNNNNNNNNNNNNNNNNNNCCNNN5'
                          PAM
```

or

```
5'NNNCCNNNNNNNNNNNNNNNNNNNNNNN3'
3'NNNGGNNNNNNNNNNNNNNNNNNNNNN5'
   PAM
```

Take the 20 nucleotides upstream of the NGG PAM. That is the + strand (green) and the complementary the – (blue). Since the *rrk1* promoter/leader system uses RNA Pol II, there is no need to avoid stretches of T that would terminate transcription by RNA Pol III. Target sites can be quite variable in terms of cleavage efficiency(*1*, *2*), so a good approach is to choose several targets around the intended mutation site and test them for cleavage in the presence of Cas9 (for example measuring the decrease in colony number as compared to an untargeted or mistargeted gRNA). A good target (decreases of 1/100 to 1/1000 in colony count) usually performs well for mutagenesis.

Once the target is chosen, design a HR mutagenesis donor template that carries the desired mutation and a mutation in the last 5-10 N in the target, or the PAM, that will abrogate cleavage. Ideally the desired mutation will be in the cleavage target/PAM, but if this is not possible, a silent mutation in the target will induce the inclusion of the desired mutation as long as the latter is reasonably close to the cleavage site.

2. **Targeting sequence cloning**

2.1. <u>By ligation of oligonucleotide duplexes</u> (requires CspCI digestion).

Order oligos with the following overhangs:

```
  5'NNNNNNNNNNNNNNNNNNNNgt3'
3'ttNNNNNNNNNNNNNNNNNNNN5'
```

Digest pMZ283 with CspCI (don't forget it needs AdoMet), dephosphorylate and gel purify.

Phosphorylate 1μl of 10μM oligo, each separately, with PNK. Mix 1:1 and heat to 95 for 3 minutes followed by gradual cooling (you can simply take the metal block out of the heater and leave it at RT, it will cool down gradually) to around 30C.

Ligate with the vector at 20x molar excess and transform.

You can use the + oligo together with M13F (TGT AAA ACG ACG GCC AGT) to check the colonies by PCR, should give 223 bp. Usually all positive clones are correct but sequencing them (M13F primer) will confirm this.

2.2. By PCR with overlapping primers

CspCI is an extremely fickle enzyme, and some batches that pass QC on lambda DNA do not efficiently cleave the placeholder in the gRNA cassette(3), and alter the electrophoretic mobility of the digestion products(4). While some improvement can be obtained by heat-inactivating the reaction before electrophoresis(4), inefficient digestion can lead to unproductive ligations. Rather than hunting for a working batch of CspCI, targeted gRNAs can be cloned with PCR approaches that sidestep these problems.

2.2.1. *Rapid single fragment method* (From Yuhei Goto, Yoshi Watanabe lab)

Order oligos with the following sequence (as before, green Ns signify the + sequence of the gRNA, blue Ns signify the reverse complement or – sequence)

gRNA_F 5′NNNNNNNNNNNNNNNNNNNgttttagagctagaaatagc 3′

gRNA_R 5′NNNNNNNNNNNNNNNNNNNttcttcggtacaggttatg 3′

PCR mixture

Oligo F (1uM)	2ul
Oligo R (1uM)	2ul
template plasmid (~100pg/ul)*	1ul
2x PrimestarMax (Takara)	5ul
total	10ul

PCR program

98°C 10sec
52°C 15sec
72°C 2min
35 cycles

Check 5 ul by agarose electrophoresis.

Transform 0.5ul directly into DH5α competent cells.

If PCR is successfully amplified, almost all colonies have the designed targeting sequence and unexpected mutations in other sites are rare.

Template plasmid amount is quite important for this method. More than 200pg of template plasmid often causes contamination of the transformation with PCR product, resulting in a majority of clones containing the empty template plasmid.

Too much PCR product for transformation decreases the efficiency, probably due to the toxicity. 0.5ul or rather less PCR product is enough.

2.2.2. *Two fragment method*

If using the combined gRNA/Cas9 vector, one may want to avoid PCR amplification of the Cas9 expression cassette. In this case, the gRNA cassette may be amplified in two fragments with the primers used in 2.1, plus the primers below:

BB_R `5'cgaggatttcgaccaggata 3'`

BB_F `5'tgtggaattgtgagcggata 3'`

BB_R/gRNA_F, fragment size 1454

BB_F/gRNA_R, fragment size 461

These two fragments can be cloned directly by gibson assembly with the gel purified plasmid backbone digested with StuI and SpeI (pMZ283 fragment size 4631, pMZ374 fragment size 9800). Alternatively, they can be gel purified and joined together in a second PCR reaction with BB_R and BB_F, digested with StuI and SpeI (provided that the targeting sequence cloned doesn't add a new site for these two enzymes) and ligated together with the plasmid backbone digested and isolated as above.

1. R. Chari, P. Mali, M. Moosburner, G. M. Church, Unraveling CRISPR-Cas9 genome engineering parameters via a library-on-library approach. *Nature Methods* (2015), doi:10.1038/nmeth.3473.

2. J. G. Doench *et al*., Rational design of highly active sgRNAs for CRISPR-Cas9-mediated gene inactivation. *Nature Biotechnology*. **32**, 1262–1267 (2014).

3. Maria Rodriguez Lopez (Bahler lab) Pers. comm.

4. Nicola Zillio Pers. comm.