Box S4: Manual gRNA selection

Copy the sequence of the open reading frame of the gene of interest (for example from the *Saccharomyces* genome database) with the flanking regions (500-1000 bp on each side of the locus) into a DNA manager software.

Select target sequences by selecting the 20 nucleotides before a PAM (NGG) sequence. In this example, part of the *CAN1* open reading frame is selected.

```
ctttctccag catttggtgc ggccaatggt tacatgtatt ggttttcttg ggcaatcact
gaaagaggtc gtaaaccacg ccggttacca atgtacataa ccaaaagaac ccgttagtga
.................CAN1.........................................
      >>......gRNA1.......>>
                          >>......gRNA2.......>>
         <<......gRNA3.......<<
                           >>......gRNA4.......>>
                        <<......gRNA5.......<<
```

gRNA1 (5' → 3'): CCAGCATTTGGTGCGGCCAA
gRNA2 (5' → 3'): GTTACATGTATTGGTTTTCT
gRNA3 (5' → 3'): CCATTGGCCGCACCAAATGC
gRNA4 (5' → 3'): TTACATGTATTGGTTTTCTT
gRNA5 (5' → 3'): AAACCAATACATGTAACCAT

Use BLAST (https://blast.ncbi.nlm.nih.gov/) to find potential off-targets. Any sequence with >16/20 identities that is followed by a PAM sequence is considered an off-target. Discard any sequence with off-targets.
Discard any sequences containing a stretch of six or more Ts.

Optional: calculate AT content for each sequence:
gRNA1: CCAGCATTTGGTGCGGCCAA 8/20, AT-score of 0.4
gRNA2: GTTACATGTATTGGTTTTCT 14/20, AT-score of 0.7
gRNA3: CCATTGGCCGCACCAAATGC 8/20, AT-score of 0.4
gRNA4: TTACATGTATTGGTTTTCTT 15/20, AT-score of 0.75
gRNA5: AAACCAATACATGTAACCAT 14/20, AT-score of 0.7

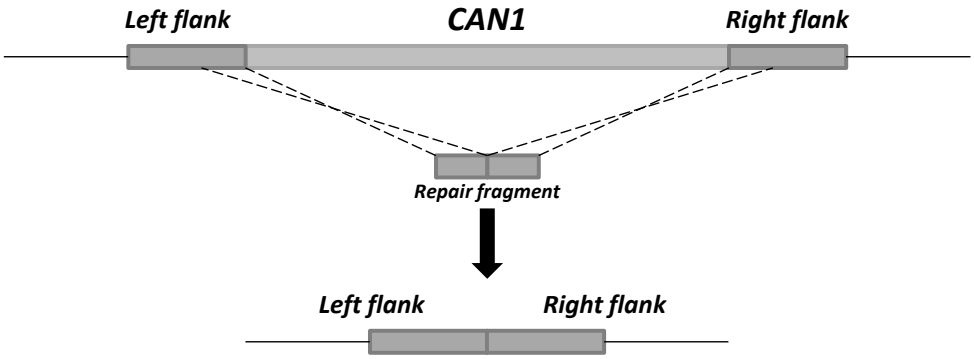
Optional: predict the RNA folding to calculate the secondary structure score (for example via http://rna.tbi.univie.ac.at/cgi-bin/RNAWebSuite/RNAfold.cgi with the folding temperature set to 30°C).
As input, provide the 20bp gRNA target sequence followed by the sequence encoding the structural RNA:

20 bp + GTTTTAGAGCTAGAAATAGCAAGTTAAAATAAGGCTAGTCCGTTATCAACTTGAAAAAGT
GGCACCGAGTCGGTGGTGCTTTTTTT

The RNA folding score is calculated by dividing the number of the 20 target sequence encoding bases predicted not to be involved in secondary structures by 20 (e.g. when no bases predicted to be involved in secondary structures results in a score of 20/20 = 1.0. Alternatively, when 7 bases are predicted to be involved in secondary structures, a score of 13/20 = 0.65 is given)

Box S5: Manual repair fragment design for gene deletion

Note: In this example, the target for deletion is the open reading frame of *CAN1* (leaving the promoter and terminator intact). Based on the desired genotype after transformation, the homologous sequences can be chosen further away or closer to the *cas9* restriction site.



Copy the sequence of the open reading frame of the gene of interest (for example from the *Saccharomyces* genome database) with the flanking regions (500-1000 bp on each side of the locus) into a DNA manager software.

Select the 60 nucleotides up- and downstream of the *CAN1* open reading frame and add the sequences together to make the forward primer. Take the reverse-complementary sequence (e.g. via https://www.bioinformatics.org/sms/rev_comp.html) for the reverse primer.

60 nucleotides upstream

```
gaaagtttat ttcagagttc ttcagacttc ttaactcctg taaaaacaaa aaaaaaaaaa aggcatagca atgacaaatt caaaagaaga cgccgacata
ctttcaaata aagtctcaag aagtctgaag aattgaggac atttttgttt ttttttttt tccgtatcgt tactgtttaa gttttcttct gcggctgtat
                                                                >>.................CAN1.........
```

60 nucleotides downstream

```
ttttgggaca aattttggaa tgttgtagca tagatatgac gttttattac ctttaatcac attcccacgc catttcgcat tctcaccctc ataagtcata
aaaaccctgt ttaaaacctt acaacatcgt atctatactg caaaataatg gaaattagtg taagggtgcg gtaaagcgta agagtgggag tattcagtat
...........CAN1.....................>>
```

Forward primer (5' → 3'):
TTCAGAGTTCTTCAGACTTCTTAACTCCTGTAAAAACAAAAAAAAAAAAAAAGGCATAGCAATATGACGTTTTATT
ACCTTTAATCACATTCCCACGCCATTTCGCATTCTCACCCTCATA

Reverse primer (5' → 3'):
TATGAGGGTGAGAATGCGAAATGGCGTGGGAATGTGATTAAAGGTAATAAAACGTCATATTGCTATGCCTTTTTT
TTTTTTTTGTTTTTACAGGAGTTAAGAAGTCTGAAGAACTCTGAA

Box S6: Manual repair fragment design for mutations

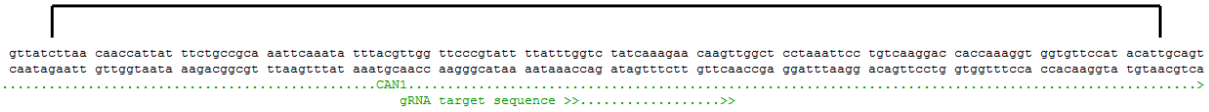
Copy the sequence of the open reading frame of the gene of interest (for example from the *Saccharomyces* genome database) with the flanking regions (500-1000 bp on each side of the locus) into a DNA manager software.

Design a gRNA of which either the target sequence or PAM sequence overlaps with the desired site for mutagenesis.

For the repair fragment, select 120 nucleotides, consisting of:

- 50 nucleotides upstream of the target sequence
- 20 nucleotides target sequence
- 50 nucleotides downstream of the target sequence

120 nucleotides



To prevent restriction by *cas9* after DNA repair, the desired mutation is introduced in the repair fragment and should disrupt the target or PAM sequence after DNA repair.

In this example, the following target+PAM sequence was used:

TGGTCTATCAAAGAACAAGTTGG

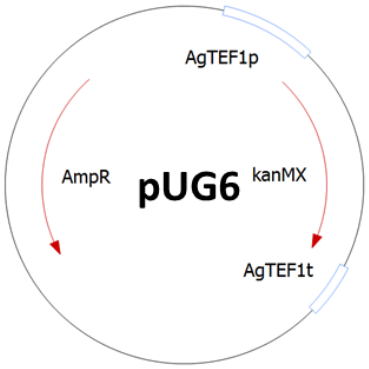
Examples of suitable repair fragments:

#1 TGGTCTATCAAAGAACAAGTT**T**G (G to T, disruption of the PAM sequence)
#2 TGGTCTATCAAAG**C**ACAAGTTGG ((A to C, disruption of the target sequence)

For example #1:
The forward primer is (5' → 3'):
ATTATTTCTGCCGCAAATTCAAATATTTACGTTGGTTCCCGTATTTTATTTGGTCTATCAAAGAACAAGTT**T**GCT
CCTAAATTCCTGTCAAGGACCACCAAAGGTGGTGTTCCATACATT

And the reverse primer is (5' → 3'):
AATGTATGGAACACCACCTTTGGTGGTCCTTGACAGGAATTTAGGAGC**A**AACTTGTTCTTTGATAGACCAAATAA
AATACGGGAACCAACGTAAATATTTGAATTTGCGGCAGAAATAAT

Box S7: Designing primers for DNA integration



In this example, the *kanMX* marker with the *Ashbya gossyppii* promoter and terminator from the plasmid pUG6 (http://www.euroscarf.de/plasmid_details.php?accno=P30114) (Güldener *et al.* 1996)) is integrated in the *CAN1* locus. When aiming to integrate multiple fragments, we refer the reader to (Kuijpers *et al.* 2013) for design of the fragments.

Design the forward and reverse primer for amplification of the DNA.

```
cgccgggtca cccggccagc gacatggagg cccagaatac cctccttgac agtcttgacg
gcggcccagt gggccggtcg ctgtacctcc gggtcttatg ggaggaactg tcagaactgc
                     >>.........................AgTEF1p........
                     >>...kanMX forward...>>


atgcgtcaat cgtatgtgaa tgctggtcgc tatactgctg tcgattcgat actaacgccg
tacgcagtta gcatacactt acgaccagcg atatgacgac agctaagcta tgattgcggc
......AgTEF1t.........................>>
                <<...kanMX reverse....<<
```

Forward primer (kanMX forward, 5' → 3'): GACATGGAGGCCCAGAATACC
Reverse primer (kanMX reverse, 5' → 3'): CAGTATAGCGACCAGCATTCAC

Attach 60 nucleotides upstream of the *CAN1* open reading frame to the forward primer:

60 nucleotides upstream

```
gaaagtttat ttcagagttc ttcagacttc ttaactcctg taaaaacaaa aaaaaaaaaa aggcatagca atgacaaatt caaaagaaga cgccgacata
ctttcaaata aagtctcaag aagtctgaag aattgaggac atttttgttt ttttttttt tccgtatcgt tactgtttaa gttttcttct gcggctgtat
                                                                        >>.................CAN1.........
```

Forward primer (5' → 3'):
TTCAGAGTTCTTCAGACTTCTTAACTCCTGTAAAAACAAAAAAAAAAAAAAAGGCATAGCAGACATGGAGGCCCAG
AATACC

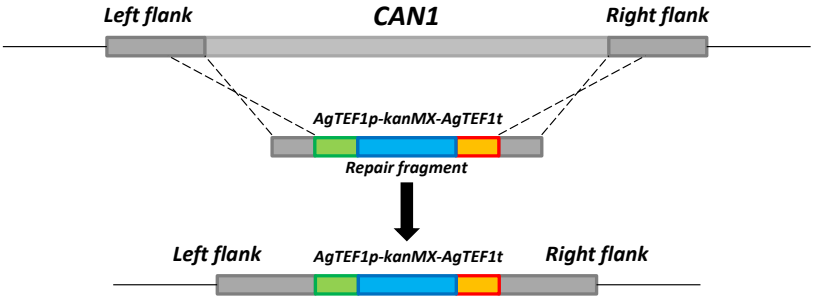
Attach 60 nucleotides downstream of the *CAN1* open reading frame to the reverse primer (make sure to use the reverse-complement for the correct orientation).

60 nucleotides downstream

```
ttttgggaca aattttggaa tgttgtagca tagatatgac gttttattac ctttaatcac attcccacgc catttcgcat tctcaccctc ataagtcata
aaaaccctgt ttaaaacctt acaacatcgt atctatactg caaaataatg gaaattagtg taagggtgcg gtaaagcgta agagtgggag tattcagtat
..........CAN1.....................>>
```

Reverse primer (5' → 3'):
CAGTATAGCGACCAGCATTCACTATGAGGGTGAGAATGCGAAATGGCGTGGGAATGTGATTAAAGGTAATAAAAC
GTCATAT

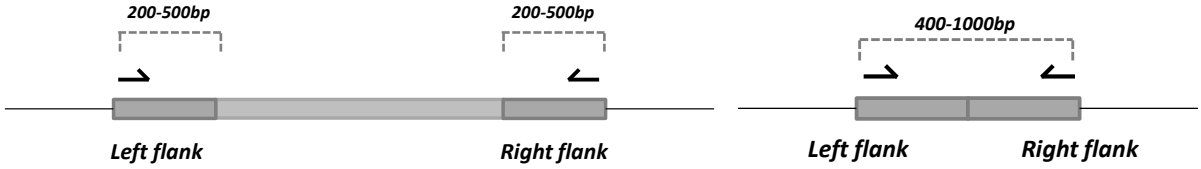
The resulting PCR fragment will have homologous flanks to the up- and downstream region of the *CAN1* open reading frame and will integrate *AgTEF1p-kanMX-AgTEF1t* into the *CAN1* locus.



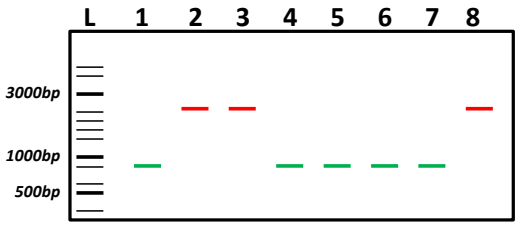
Box S8: Manual primer design for colony PCR of yeast transformants

For confirmation of successful yeast transformations, in which a gene ORF is either deleted or used as a site for integration of (heterologous) DNA, we suggest confirming both the absence of the original gene ORF and the correct integration of DNA via PCR.

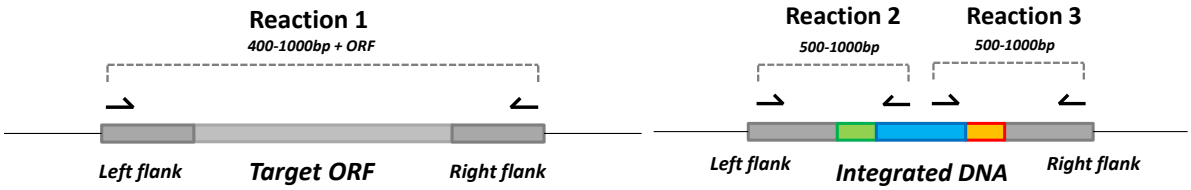
For the case of gene deletions, design primers that bind 200-500 bp outside the target ORF. This will result in a 400-1000 bp PCR product when the gene has been successfully deleted and a PCR product of 400-1000 bp + the size of the ORF when the gene is present. We recommend to take the DNA template of the parental strain along as a control.



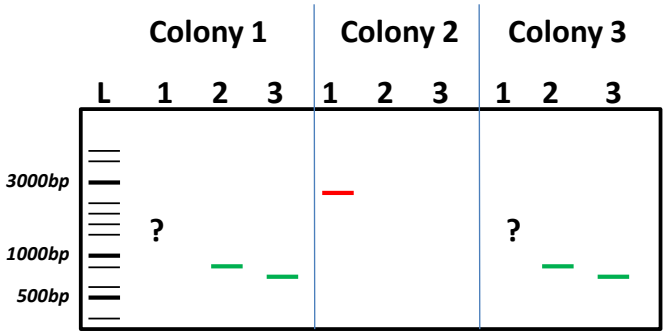
On the right: example of a typical gel when aiming for gene deletions and confirmation of 8 colonies via PCR, using primers binding outside the target ORF. The large fragments (red) correspond to colonies in which the target ORF is still present, the short fragments (green) correspond to colonies containing the gene deletion.



For gene integrations, design the same primers binding outside the target ORF, but also design primers binding inside the target construct such that the products of reaction 2 and 3 (see below) have a size of 500-1000 bp. Perform 3 PCR reactions per colony:



On the right: example of a typical gel when aiming for gene integration and confirmation of 3 colonies via PCR, using primers binding outside the target ORF and inside the integrated DNA. The large fragments (red) correspond to colonies in which the target ORF is still present, the short fragments (green) correspond to the results of reaction 2 and reaction 3 from colonies containing the gene integration. For integration of large constructs, usually no product is obtained in reaction 1 as the product is too long to be efficiently amplified via PCR (indicated by the "?" in the figure). When aiming to integrate multiple fragments, we refer the reader to (Kuijpers *et al.* 2013) for design of the confirmation PCR.



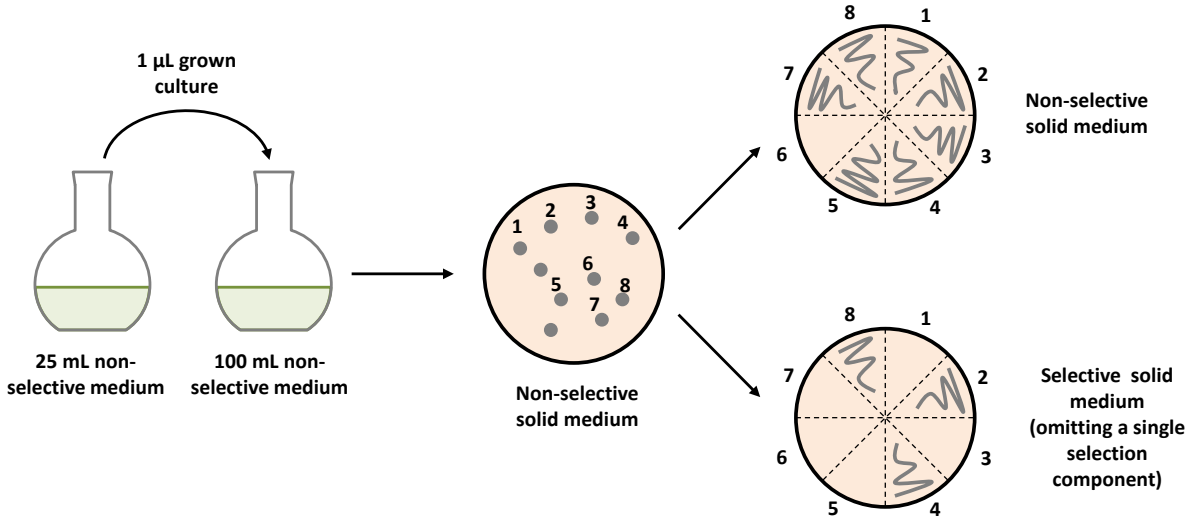
For introduction of SNPs, we recommend PCR amplification of the 1 kb region surrounding the mutation site followed by Sanger sequencing of the PCR product.

Box S9: Plasmid removal after a/multiple round(s) of transformation

Before testing the phenotype of engineered yeast strains, we recommend removal of gRNA coding plasmids. The method described here can theoretically be used for the simultaneous removal of as many plasmids as desired, but has been demonstrated to be efficient for removal of up to at least 4 plasmids.

A -80°C stock culture or single colony with the correct genetic modification is first grown in 25 mL non-selective medium. After the culture is fully grown, 1 µL of culture is transferred to 100 mL non-selective medium. This second culture ensures growth with sufficient generations (~17) to promote plasmid loss. A fraction of the culture is then plated on a non-selective plate for single colonies. After colonies are clearly visible, 8-16 single colonies are streaked to a selective and non-selective plate. The non-selective plate is used to determine that the transferred colony was viable, the selective plate indicates which colonies have lost the plasmid. When multiple plasmids are removed simultaneously, a selective plate needs to be made for each plasmid to confirm plasmid loss for each plasmid individually (example below).

Workflow:



In the workflow shown above, colony #2, #4 and #8 still contain the plasmid, colony #6 was not viable and colony #1, #3, #5 and #7 were viable and successfully lost the plasmid.

Example for removal of multiple plasmids: removal of plasmids from a strain carrying pROS10, pROS14 and pROS16 (*URA3*, *LEU2* and *HIS3*):

- Non-selective medium could be YPD or SM+uracil+leucine+histidine.
- After growing in non-selective liquid medium, each single colony is restreaked on 4 plates:
    a. SM+leucine+histidine (check for uracil auxotrophy)
    b. SM+uracil+histidine (check for leucine auxotrophy)
    c. SM+uracil+leucine (check for histidine auxotrophy)
    d. YPD or SM+uracil+leucine+histidine (confirm colony viability)
- A colony that only grows on the non-selective plate ("plate d") has lost all pROS plasmids and can be used for strain characterization or further engineering.

NOTE: Not every transformation event requires a subsequent round of plasmid removal. Exhausting all available markers in multiple transformations prior to a single round of plasmid removal results in the most time-efficient gene editing.