SUPPORTING INFORMATION

Full input query submitted to the AMUSER web server for experimental demonstration.

>GFPmut3

ATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGTGATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGG

GTGAAGGTGATGCAACATACGGAAAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTTGTCACTACTTTCGG

TTATGGT**[GTTCAA=CTGAAG]**TGCTTTGCGAGATACCCAGATCATATGAAACAGCATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAG

GAAAGAACTATATTTTTCAAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTTAATAGAATCGAGTTAAAAG

GTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAATTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAA

TGGAATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGACCATTATCAACAAAATACTCCAATTGGCGATGGC

CCTGTCCTTTTACCAGACAACCATTACCTGTCC**[ACA=TAC]**CAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTTCTTG

AGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAA

>pZE21

CCTCGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCCATGGTACGCGTGCTAGAGGCATCAAATAAAACGAAAGG

CTCAGTCGAAAGACTGGGCCTTTCGTTTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGCCCTAGACCTAGGGCGTTCG

GCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCCACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAG

CAAAAGGCCAGGAACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCATCACAAAAATCGACGCTCAAGTCAGA

GGTGGCGAAACCCGACAGGACTATAAAGATACCAGGCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGATA

CCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGGTATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGC

TGTGTGCACGAACCCCCCGTTCAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACACGACTTATCGCCACTGG

CAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGCGGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGAC

AGTATTTGGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCCGGCAAACAAACCACCGCTGGTAGCGGTGGT

TTTTTTGTTTGCAAGCAGCAGATTACGCGCAGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGGAACGAAA

ACTCACGTTAAGGGATTTTGGTCATGACTAGTGCTTGGATTCTCACCAATAAAAAACGCCCGGCGGCAACCGAGCGTTCTGAACAAATCCAGATGGA

GTTCTGAGGTCATTACTGGATCTATCAACAGGAGTCCAAGCGAGCTCTCGAACCCCAGAGTCCCGCTCAGAAGAACTCGTCAAGAAGGCGATAGAAG

GCGATGCGCTGCGAATCGGGAGCGGCGATACCGTAAAGCACGAGGAAGCGGTCAGCCCATTCGCCGCCAAGCTCTTCAGCAATATCACGGGTAGCCA

ACGCTATGTCCTGATAGCGGTCCGCCACACCCAGCCGGCCACAGTCGATGAATCCAGAAAAGCGGCCATTTTCCACCATGATATTCGGCAAGCAGGC

ATCGCCATGGGTCACGACGAGATCCTCGCCGTCGGGCATGCGCGCCTTGAGCCTGGCGAACAGTTCGGCTGGCGCGAGCCCCTGATGCTCTTCGTCC

AGATCATCCTGATCGACAAGACCGGCTTCCATCCGAGTACGTGCTCGCTCGATGCGATGTTTCGCTTGGTGGTCGAATGGGCAGGTAGCCGGATCAA

GCGTATGCAGCCGCCGCATTGCATCAGCCATGATGGATACTTTCTCGGCAGGAGCAAGGTGAGATGACAGGAGATCCTGCCCCGGCACTTCGCCCAA

TAGCAGCCAGTCCCTTCCCGCTTCAGTGACAACGTCGAGCACAGCTGCGCAAGGAACGCCCGTCGTGGCCAGCCACGATAGCCGCGCTGCCTCGTCC

TGCAGTTCATTCAGGGCACCGGACAGGTCGGTCTTGACAAAAAGAACCGGGCGCCCCTGCGCTGACAGCCGGAACACGGCGGCATCAGAGCAGCCGA

TTGTCTGTTGTGCCCAGTCATAGCCGAATAGCCTCTCCACCCAAGCGGCCGGAGAACCTGCGTGCAATCCATCTTGTTCAATCATGCGAAACGATCC

TCATCCTGTCTCTTGATCAGATCTTGATCCCCTGCGCCATCAGATCCTTGGCGGCAAGAAAGCCATCCAGTTTACTTTGCAGGGCTTCCCAACCTTA
CCAGAGGGCGCCCCAGCTGGCAATTCCGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTC
TTCACCTCGAGTCCCTATCAGTGATAGAGATTGACATCCCTATCAGTGATAGAGATACTGAGCACATCAGCAGGACGCACTGACCGAATTCATTAAA
GAGGAGAAAGGTACCG

Table 1. Primers as calculated when submitting the input above.

Name	**Description**	**Sequence (5'-3')**
oGEN118	GFPmut3 - part 1 of 3 fw	ACCGATGCGUAAAGGAGAAGAACTTTTCA
oGEN119	GFPmut3 - part 1 of 3 rv	AGCACTUCAGACCATAACCGAAAGTAGT
oGEN120	GFPmut3 - part 2 of 3 fw	AAGTGCUTTGCGAGATACCCAGATC
oGEN121	GFPmut3 - part 2 of 3 rv	AGATTGGUAGGACAGGTAATGGTTGTCT
oGEN122	GFPmut3 - part 3 of 3 fw	ACCAATCUGCCCTTTCGAAAGATCCC
oGEN123	GFPmut3 - part 3 of 3 rv	AGGTTAUTTGTATAGTTCATCCATGCCA
oGEN107	FW_pZE21_[Tm:56.9]	ATAACCUCGAGGTCGACGGTATCGA
oGEN108	RV_pZE21_[Tm:54.1]	ACGCATCGGUACCTTTCTCCTCTTTAATGAATTC

primer details for GFPmut3 - part 1 of 3

forward primer: ACCGATGCGUAAAGGAGAAGAACTTTTCA

* Tm: 48.0C - in optimal range (55-72)?	...NO
* GC content: 31.58% - in optimal range (40-60)?	...NO
* GC clamp present at 3' end?	...YES
* more than 3 G/C out of last 5 bases at 3' end?	...NO
* risk of primer dimer formation in primer pair?	...NO
* risk of intra-primer homology (secondary structures)?	...NO
* presence of polyN stretches?	...YES

reverse primer: AGCACTUCAGACCATAACCGAAAGTAGTG

* Tm: 49.9C - in optimal range (55-72)?	...NO
* GC content: 42.11% - in optimal range (40-60)?	...YES
* GC clamp present at 3' end?	...YES
* more than 3 G/C out of last 5 bases at 3' end?	...NO
* risk of primer dimer formation in primer pair?	...NO
* risk of intra-primer homology (secondary structures)?	...NO
* presence of polyN stretches?	...NO

Tm of primers within 2C of each other? ...YES

Figure 1. Primer details from the AMUSER web server results page. The desired properties are color-coded in green and undesired properties are shown in red.

details of final construct after cloning (circular, length: 2964 bases):

GFPmut3 - part 1 of 3 | insert1 | GFPmut3 - part 2 of 3 | insert2 | GFPmut3 - part 3 of 3 | pZE21

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1       ATGCGTAAAGGAGAAGAACTTTTCACTGGAGTTGTCCCAATTCTTGTTGAATTAGATGGT
61      GATGTTAATGGGCACAAATTTTCTGTCAGTGGAGAGGGTGAAGGTGATGCAACATACGGA
121     AAACTTACCCTTAAATTTATTTGCACTACTGGAAAACTACCTGTTCCATGGCCAACACTT
181     GTCACTACTTTCGGTTATGGTCTGAAGTGCTTTGCGAGATACCCAGATCATATGAAACAG
241     CATGACTTTTTCAAGAGTGCCATGCCCGAAGGTTATGTACAGGAAAGAACTATATTTTTC
301     AAAGATGACGGGAACTACAAGACACGTGCTGAAGTCAAGTTTGAAGGTGATACCCTTGTT
361     AATAGAATCGAGTTAAAAGGTATTGATTTTAAAGAAGATGGAAACATTCTTGGACACAAA
421     TTGGAATACAACTATAACTCACACAATGTATACATCATGGCAGACAAACAAAAGAATGGA
481     ATCAAAGTTAACTTCAAAATTAGACACAACATTGAAGATGGAAGCGTTCAACTAGCAGAC
541     CATTATCAACAAAATACTCCAATTGGCGATGGCCCTGTCCTTTTACCAGACAACCATTAC
601     CTGTCCTACCAATCTGCCCTTTCGAAAGATCCCAACGAAAAGAGAGACCACATGGTCCTT
661     CTTGAGTTTGTAACAGCTGCTGGGATTACACATGGCATGGATGAACTATACAAATAACCT
721     CGAGGTCGACGGTATCGATAAGCTTGATATCGAATTCCTGCAGCCCGGGGGATCCCATGG
781     TACGCGTGCTAGAGGCATCAAATAAAACGAAAGGCTCAGTCGAAAGACTGGGCCTTTCGT
841     TTTATCTGTTGTTTGTCGGTGAACGCTCTCCTGAGTAGGACAAATCCGCCGCCCTAGACC
901     TAGGGCGTTCGGCTGCGGCGAGCGGTATCAGCTCACTCAAAGGCGGTAATACGGTTATCC
961     ACAGAATCAGGGGATAACGCAGGAAAGAACATGTGAGCAAAAGGCCAGCAAAAGGCCAGG
1021    AACCGTAAAAAGGCCGCGTTGCTGGCGTTTTTCCATAGGCTCCGCCCCCCTGACGAGCAT
1081    CACAAAAATCGACGCTCAAGTCAGAGGTGGCGAAACCCGACAGGACTATAAAGATACCAG
1141    GCGTTTCCCCCTGGAAGCTCCCTCGTGCGCTCTCCTGTTCCGACCCTGCCGCTTACCGGA
1201    TACCTGTCCGCCTTTCTCCCTTCGGGAAGCGTGGCGCTTTCTCATAGCTCACGCTGTAGG
1261    TATCTCAGTTCGGTGTAGGTCGTTCGCTCCAAGCTGGGCTGTGTGCACGAACCCCCCGTT
1321    CAGCCCGACCGCTGCGCCTTATCCGGTAACTATCGTCTTGAGTCCAACCCGGTAAGACAC
1381    GACTTATCGCCACTGGCAGCAGCCACTGGTAACAGGATTAGCAGAGCGAGGTATGTAGGC
1441    GGTGCTACAGAGTTCTTGAAGTGGTGGCCTAACTACGGCTACACTAGAAGGACAGTATTT
1501    GGTATCTGCGCTCTGCTGAAGCCAGTTACCTTCGGAAAAAGAGTTGGTAGCTCTTGATCC
1561    GGCAAACAAACCACCGCTGGTAGCGGTGGTTTTTTTGTTTGCAAGCAGCAGATTACGCGC
1621    AGAAAAAAAGGATCTCAAGAAGATCCTTTGATCTTTTCTACGGGGTCTGACGCTCAGTGG
1681    AACGAAAACTCACGTTAAGGGATTTTGGTCATGAGTAGTGCTTGGATTCTCACCAATAAA
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Figure 2. Construct details from the AMUSER web server results page. Sequence of final construct after site-directed mutagenesis and assembly. Colors indicate different parts and assembly regions.

Protocol for PCR-based USER cloning

Adapted and modified from [1]

Reagents

- Uracil primers as designed using the AMUSER web server (http://www.cbs.dtu.dk/services/AMUSER/)
- Phusion U Hot Start DNA Polymerase (Thermo Scientific, F-555S)
  Note: Other polymerases such as PfuX7[2] and PfuTurbo Cx Hotstart DNA Polymerase (Agilent Technologies, 600410) are also compatible.
- DpnI restriction endonuclease
- USER Enzyme (NEB, M5505)
- Chemically competent *E.coli*

Procedure

1. Amplify target sequences by PCR using uracil primers following the Polymerase manufactures instructions.
2. Purify PCR products of correct size.
3. Remove template DNA by mixing linearized plasmid and fragments in a PCR tube with DpnI enzyme, buffer and water as follows and incubate at 37° C for 15 min.

1-7 fragments	~1 pmol combined, equimolar amounts
DpnI buffer (10X)	1 µl
DpnI enzyme	1 µl
Deionized H_2O	adjust to 10 µl

4. Create 3' overhangs by adding 1 µl of USER Enzyme and incubate for 15 min at 37° C.
5. Allow DNA to assemble by incubating for 15 min at room temperature.
6. Directly transform chemically competent *E.coli* cells with 1-5 µl of assembly mixture (ligation will be performed *in vivo* during replication) following the manufactures instructions.

References

(1) Bitinaite, J., and Nichols, N. M. (2009) DNA cloning and engineering by uracil excision. *Curr. Protoc. Mol. Biol. Chapter 3*, Unit 3.21.

(2) Nørholm, M. H. H. (2010) A mutant Pfu DNA polymerase designed for advanced uracil-excision DNA engineering. *BMC Biotechnol. 10*, 21.