Megaprime Mutagenesis

NOTE 1: The protocol utilizes two rounds of PCR to derive a point-mutated product. It is recommended to use a high-fidelity enzyme (eg. Denville SurePol) to reduce the possibility of cloning a randomly mutated product (the protocol uses 70 rounds of PCR – the chance of a random mutation event is higher if you use a low-fi enzyme). However I have also used Denville TaqPro; while reported as low fidelity, according to Tim the error rate is $1.5x10^{-5}$ after 70 cycles, or ≈ $1x10^{-6}$/cycle.

Requirements

Template: Sometimes you'll have a cloned template available. This is handy but not necessary – I've made mutants from genomic DNA (although this is probably not optimal).
Primers: You need 3: (i) forward, (ii) reverse, and (iii) mutagenic (as either F or R – it's better to use shorter megaprimers so this will influence the orientation of the mutagenic primer (F or R). **NB**. The mutation has to be in the center of the primer. Also it is sometimes possible to create or destroy a restriction enzyme site (this helps screening).

Standard rules for PCR apply (concentrations, cleanliness, etc) - also note that the larger the megaprimer, the more difficult it will be to work with.

Protocol

(1) **Round 1 PCR conditions** - creation of Megaprimer (Denville SurePol)

Mix on ice (this is for a 50µl Rxn)-

Plasmid template (or genomic DNA)	1 µl
ddH_2O	10 µl

Denature (5 min 94°C), then place on ice 1 min (variation of a hot start)

Mix on ice (should be ready to add)

2.5 µM primer 1	5 µl
2.5 µM primer 2 - **mutagenic**	5 µl
2.5 mM dNTP mix	5 µl
10 x Sure Pol Buffer	5 µl
Denville SurePol	0.5 µl
ddH_2O	to 50 µl final

PCR will be 35 cycles

35 cycles - T_1 30s 94°C; T_2 30s 50-60°C; T_3 1-2m 72°C
1 cycle – 5 min 72°C
1 cycle - ∞ 4°C

Note: The recommended length of T_3 (extension step) for SurePol is 1 minute per kB DNA.

(2) Run product on a 1 - 1.5% agarose gel and purify (this is essential). Elute in as small a volume as feasible (usually 25µl, but this depends on product abundance). This is now used as a 10x Megaprimer.

(3) **Round 2 PCR conditions** - creation of Megaprimer (Denville SurePol)

Mix on ice (this is for a 50µl Rxn)-

Plasmid template (or genomic DNA)	1 µl
10 x **Megaprimer**	5 µl
ddH_2O	10 µl

Denature (5 min 94°C), then place on ice 1 min (variation of a hot start)

Mix on ice (should be ready to add)

2.5 µM primer 3	5 µl
2.5 mM dNTP mix	5 µl
10 x Sure Pol Buffer	5 µl
Denville SurePol	0.5 µl
ddH_2O	to 50 µl final

PCR will be 35 cycles

35 cycles - T_1 30s 94°C; T_2 30s 50-60°C; T_3 2 - 4m 72°C

1 cycle – 5 min 72°C

1 cycle - ∞ 4°C

(4) Run product on a resolving gel and purify (preferred method depends on the size of the product). Resuspend / elute product in as small a volume as feasible.

(5) Clone away

NB. For simplicity we usually clone into the PCR-Topo vector

Advantages: **very** high efficiency, Kan^r (useful since it is likely that your template (if a plasmid) is Amp^r ; thus template contamination is not an issue).

(6) Restriction enzyme digest (if possible) or sequence to confirm the desired mutation. If automated sequencing remember that we usually send in sets of 8. I usually send 2 independent clones for sequencing of each mutation (just in case).

NOTES :

(i) Efficiency is usually >90%

(ii) When designing the flanking primers keep in mind downstream applications – add useful RE sites, etc ….

(iii) When designing the mutagenic primer remember it has to be optimally designed for PCR at BOTH ends (never begin or end with an A or T). Also ensure at least 8 – 10 bases WT flank around the mutation(s).

Variant of protocol used in:

Keogh *et al* (2003) *Mol Cell Biol* **23:**7005-18.
Keogh *et al* (2006) *Genes Dev* **20:**660-5
Keogh *et al* (2006) *Nature* **439:**497-501

Yeast strains, genetic manipulations and media

Yeast plasmids were based on the pRS series (66). For mutational analysis of Bur1, a 2.45kB fragment containing the *BUR1* ORF (no stop codon) and ≈400bp of 5' promoter region was PCR amplified from *S. cerevisiae* genomic DNA using Pfu Polymerase and oligos Bur1 5'SacI(N) (GATCGAGCTCCCGAGAAATCA GCCGTTGG) and Bur1 3'FL Bam (CTAGGGATCCATATAGATCTGCAATATCA CTATTTTGG). The resulting product was gel purified and cloned in frame with an $(HA)_3$ epitope tag and SSN6 terminator at the carboxyl terminus. This product was transferred to pRS315 to create pRS315-BUR1-HA_3.
Point mutations in Bur1 (T70A, E107Q, D213A, T240A, T240D)) were generated using PCR-mediated site-directed mutagenesis (29, 34). Primer Bur1 5'SacI(N) and the appropriate mutagenic primer (sequences available upon request) were used to amplify the 5' end of the *BUR1* gene. The resulting PCR product was gel-purified and utilized as a 5' megaprimer in a second PCR reaction with the 3' primer Bur1 3'FL Bam. The resulting 2.45kB amplified fragments were cloned into pRS315-$(HA)_3$-SSN6 such that the mutant protein was epitope tagged at the C-terminus.