Protocol for Multi-site Mutagenesis

The Megaprimer PCR of Whole Plasmids (MEGAWHOP) method [1] was successfully used to create protein chimeras where a helix from one protein was swapped with that of another and vice versa (see Figure 1A). The swap involved mutation of 7 amino acids in a 20 residue stretch of sequence: -MXXXXMMXXXXXMXXXMXMM- where M=mutated site; X=non-mutated site

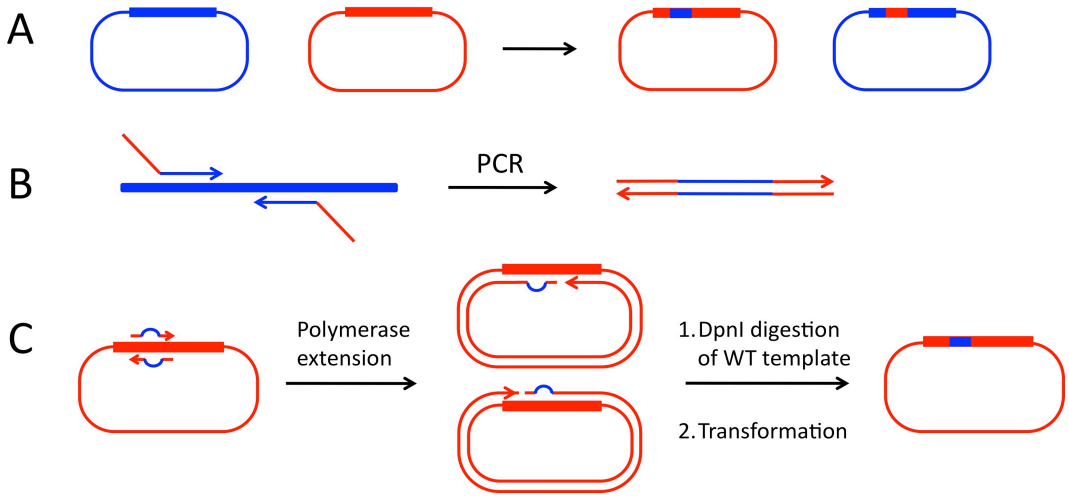

Figure 1. Schematic of mutagenesis protocol. (A) Chimeras were made from wild type plasmids (blue and red) containing genes of interest (rectangles). A 60bp region of each gene was swapped. (B) The region of interest is PCR amplified to create two complementary mutagenic primers (megaprimers). The 5' end of each oligo includes a region of overlap with the destination plasmid (shown in red). (C) Whole plasmid PCR using megaprimers creates DNA containing desired mutations. After DpnI digestion of the WT template, the reaction is transformed into bacteria and clones are analyzed by DNA sequencing.

The WT genes were ~2kb and the template plasmids were relatively large (~7.5kb) and GC rich. These factors made it a challenging system for mutagenesis. Although the efficiencies of the MEGAWHOP reactions were low (2 hits in 6 and 1 hit in 10 sequenced clones), all 7 amino acid mutations were inserted simultaneously. Attempts to introduce the 7 mutations in two rounds, 3 and 4 mutations at a time, via 2-stage Quikchange mutagenesis [2] were unsuccessful.

The megaprimers were ~100bp and were generated by PCR of the appropriate template (see Figure 1B). We intended to use 15bp of complementarity to the destination vector on either side of the mutated region but ended up having much less (11 or 5 bp of overlap) on one of the sides due to errors in primer design. In future, it would be preferable to use a larger overlap on either side (20bp) [3]. Vent polymerase was used for PCR amplification, and the megaprimer product was gel purified and isolated using a QIAQuick gel extract kit. The DNA was eluted from the QIAQuick membrane with ~30uL water and quantified by absorbance at 260nm.

The MEGAWHOP reaction (see Figure 1C) largely followed the Miyazaki protocol [4] which 'guarantees a good outcome with a 500-1000bp megaprimer and a 3-7 kbp template plasmid'. The published protocols were for a 3.5kbp plasmid and ~750bp megaprimer and used 50ng template, 0.2ug megaprimer, with no additives. These conditions were tried initially but did not yield transformants. A reaction where the molar ratios of template to megaprimer were the kept the same as the Miyazaki protocol was also unsuccessful.

In optimizing conditions, the amount of template (50ng, 100ng), amount of megaprimer (0.2ug, 0.5ug), additives (glycerol, DMSO), and type of DNA polymerase (Pfu Turbo, VAPRase) were varied. The best results were obtained using 100ng template and 0.5ug megaprimer. VAPRase, a homemade version of Phusion DNA polymerase (NEB) that is sold by the Vandy MBRC Core, gave more transformants than Pfu Turbo, and the cycling times were much shorter (2.5 vs. 4hrs). The addition of 5% glycerol led to an increased the number of transformants. Addition of 3% DMSO, which

is recommended for extension of GC rich templates when using Phusion, gave ~10-fold more transformants than 5% glycerol.

The denaturation and extension temperatures used for PCR were the typical parameters for Phusion. For Phusion, extension times of 15 sec per 1kbp for low complexity DNA and 30 sec per 1kbp for high complexity DNA templates are recommended. The annealing temperature used in the Miyazaki protocol (55°C) was successfully used for one template. For the other template, I used the annealing temperature that was optimal for generation of the megaprimer (65°C). The transformation protocol largely followed the manufacturer's instructions and resulted in 20-30 colonies per plate. Use of commercial XL-10 Gold ultracompetent cells led to a significantly larger number of transformants compared to in-house XL-1 Blues. Despite DpnI digestion, the number of wild type transformants was quite high, and sequencing of a number of colonies was necessary in order to find a hit. In a couple of cases, mutations outside of the targeted region were observed[3], so full gene sequencing is recommended.

I. MEGAWHOP Reaction Mix (50uL total volume):

- 5uL VAPRase buffer (10X)
- 1.5uL DMSO
- 4uL dNTPs (2.5mM)
- 100ng template plasmid
- 0.5ug megaprimer
- 1U VAPRase (4U/uL)
- Autoclaved H_2O to 50uL

II. MEGAWHOP PCR amplification program:

1. 98°C 30 sec
2. 98°C 10 sec
3. 55°C 30 sec
4. 72°C 3 min 45sec (Depends on plasmid size. Use 30s/kb for high complexity DNA template)
5. Go to step 2 for 24 cycles
6. 4°C hold

III. DpnI Digest:

Add 20U (1uL) of DpnI (NEB) directly into the PCR reaction. Mix well. Incubate at 37°C for 2-3hrs.

IV. Transformation XL-10 Gold ultracompetent cells (Agilent Technologies):

1. Add 2uL of the DpnI-treated mixture to 50uL XL-10 cells.
2. Ice 30 min.
3. Heat shock 42°C 30 sec
4. Ice 2 min.
5. Add 225uL warmed SOC medium
6. Incubate 37°C with shaking 1hr
7. Plate 200uL on warmed LB plate containing appropriate antibiotic

References

[1] Miyazaki, K. (2011) MEGAWHOP Cloning A Method of Creating Random Mutagenesis Libraries via Megaprimer PCR of Whole Plasmids. *Methods Enzymol* 498, 399-406

[2] Wang, W. and Malcolm, B.A. (1999) Two-stage PCR protocol allowing introduction of multiple mutations, deletions and insertions using QuikChange Site-Directed Mutagenesis. *Biotechniques* 26, 680-682

[3] Geiser, M., *et al*. (2001) Integration of PCR fragments at any specific site within cloning vectors without the use of restriction enzymes and DNA ligase. *Biotechniques* 31, 88-92

[4] Miyazaki, K. (2003) Creating random mutagenesis libraries by megaprimer PCR of whole plasmid (MEGAWHOP). *Methods Mol Biol* 231, 23-28