Using QCM to introduce two or more mutations in one reaction

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(e.g. changing a His-tag position from N-term. to C-term, multiple nucleotide mutations at two different positions on a plasmid, double deletions, etc.)

This method is based on a 2-step PCR. In the first step, the region between the two regions to be mutated is amplified. In the second step, the whole plasmid gets amplified, using the PCR product from the first step as primers.

This method works surprisingly good, however, careful primer design is important for the QCM to work. For addition of tags (e.g. a 6-His tag), having at least 18 nt upstream and ~ 25 nt downstream of the tag sequence are usually sufficient (if their GC content is about normal). Primers with multiple nucleotide substitutions have to vary in length, acc. to the amount of changed nucleotides. I usually try to have ~ 30 matching nucleotides in the primers.

I use KOD extreme as DNA polymerase for these reactions. However, several other DNAPs (like Phusion, NEB Q5, etc.) work just as well.

One 50 µl rxn contains:

25 µl 2x KOD reaction buffer
30 ng of plasmid
250 nM fw primer
250 nM rv primer
10 µl 2 mM dNTPs
1 µl KOD extreme
H_2O to 50 µl

For a ~ 680 bp gene on a ~ 5 kb (total) plasmid, I use this program:

1. 95 C 2min
2. 98 C 1 sec
3. 55 C 15 sec
4. 68 C 1 min
   repeat steps 2-4 17x
5. 98 C 10 sec
6. 68 C 14 min
   repeat steps 5-6 17x
7. 68 C 6 min
8. 4 C ∞

EtOH precipitate PCR rxn, resuspend in 30 µl H2O
DpnI digest as in the standard QCM protocol

EtOH precipitate and resuspend in 25 µl H2O
Transform 1 µl into 25 µl of DH10B cells