Background info

This protocol is useful while working with short coding DNA sequences (i.e. up to 30 aa). For longer coding sequences, we recommend you to employ the KAPPA protocol also available in our website. The length limitation comes from the oligonucleotide synthesis; ask your oligonucleotide provider for scale synthesis and purification available. We routinely order either HPLC or SDS-PAGE purification.
The oligonucleotides needed are not fully complementary sequences: once they are annealed, the double-strand DNA contains single strand ends which are complementary to overhang sequences obtained after restriction enzyme digest.

Protocol

Oligo annealing

1. Prepare oligonucleotide stock solution @ 100uM.
2. Mix sense and antisense oligonucleotides in 1xMRB:

   12.5uL sense oligonucleotide
   12.5uL antisense oligonucleotide
   5.0uL 10xMRB (Medium salt Restriction Buffer)
   20.0uL H_2O (PCR grade)
   (final concentration: 25uM ds-oligo)
3. Incubate for 5min @ 95C
4. Let cool it down to room temperature (it takes 2-3 hours).
5. Make a 0.1uM working solution of ds-oligo in 1xHRB.

   2uL 25uM ds-oligo
   25uL 10xMRB
   473uL H_2O (PCR grade)

   Note: Your overhang ds-oligonucleotide stock and working solutions are ready. Keep them at -20C and do not heat them again.

Standard ligation

6. Digest 0.5ug of plasmid DNA for 1-2h (after digest, do not treat with phosphatase! Phosphate groups @ 5´ DNA end are needed for ligation).
7. Load it onto a 1% low melting point agarose gel.
8. Run for 2h @ 65V.
9. Cut the agarose piece containing the digest plasmid and transfer it to a clean tube.
10. Incubate it @ 68C until agarose is melted (2-3 min should be enough).
11. Take 9uL and place it a new tube.
12. Incubate for 2-3min @ 37C.
13. Add 10uL of pre-warmed ligase mix:

    6uL H_2O (PCR grade)
    2uL 10x Ligase buffer
    2uL T4 DNA Ligase
14. Mix well and leave it @ 37C for 2-3 more minutes.
15. Take tube and place it on the bench @ room temperature.
16. Add 1uL of ds-oligo working solution.
17. Leave it @ room temperature for 1h-O/N.

    Note: The insert:vector ratio can be optimized. In our tools section you will find a tool which may be helpful.

Reagents

-10xMRB (see restriction buffer recipes).
-T4 DNA ligase and ligase buffer (we routinely employ enzymes from NEB).
-Low Melting Agarose.
-DNA running buffer (we routinely employ TAE).
-H_2O (PCR grade)