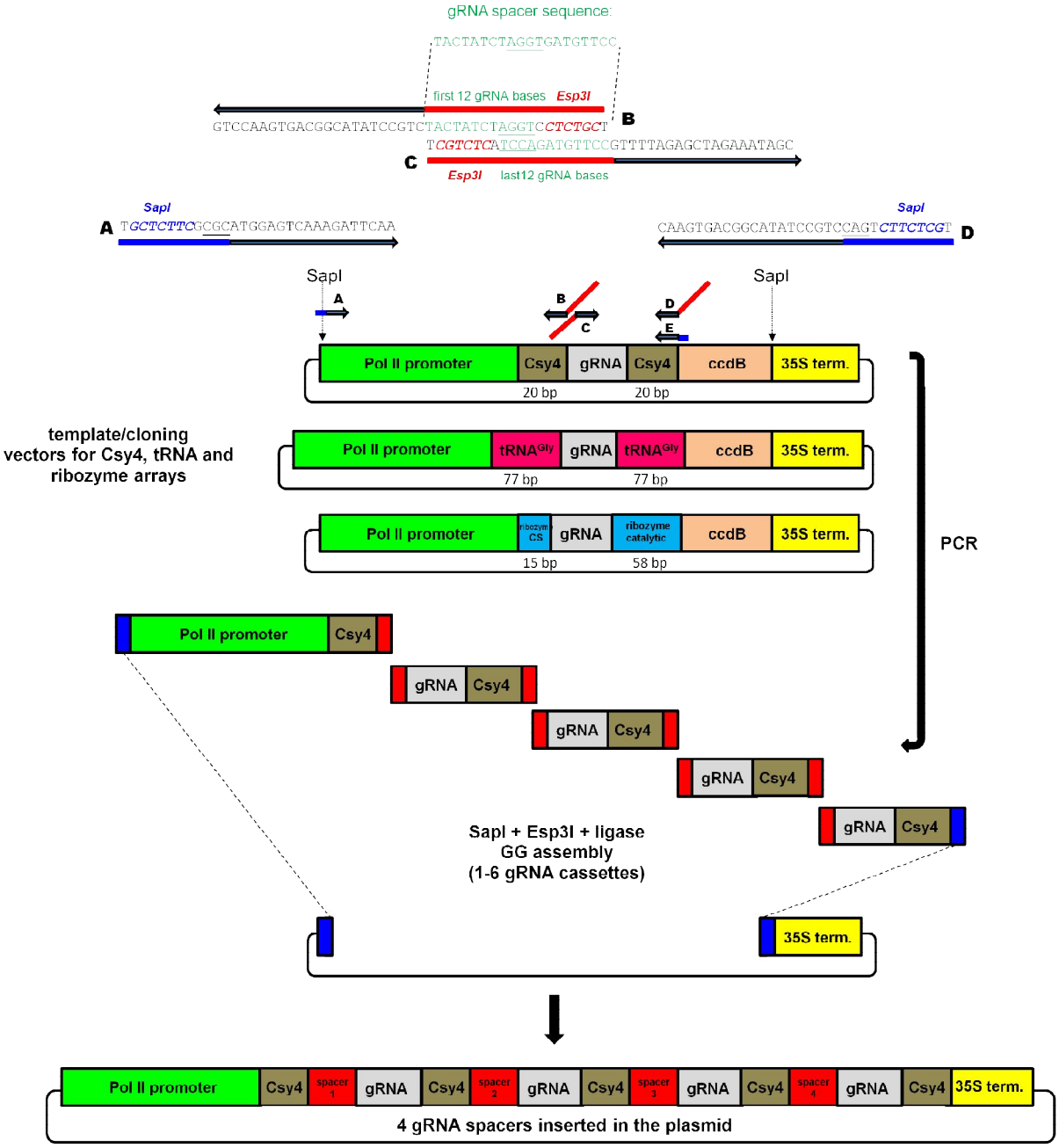
PROTOCOL 3 - DESCRIPTION

Assembly of multiple gRNA spacers into Csy4, tRNA or ribozyme arrays

This set of protocols describes cloning of multiple gRNA spacers into arrays controlled by an RNA Pol II promoter in either modular or DIRECT vectors. The approach is based on Golden Gate assembly of PCR products carrying the processing elements, gRNA repeats as well as parts of target-specific gRNA spacers providing unique sequence stretches used to design the Golden Gate junctions. First, primers are designed to amplify each gRNA part plus the promoter part as illustrated below. Inclusion of the promoter in the PCR product rather than in the plasmid backbone is based on the assumption that the use of promoters other than the ones included in the vector set might be desired. In such a case, the promoter sequence can be amplified from any template and included in the assembly, preventing the requirement to modify the vector backbone. The module/DIRECT vectors serve both as the cloning backbones and as the templates for PCR amplification of individual parts. n+1 (n = number of gRNA spacers) parts (PCR products) are assembled into the vector by Golden Gate assembly using two distinct type IIs enzymes, including SapI and one out of three other enzymes – Esp3I, BsaI or AarI, selected by the user. Cleavage by SapI will release the ccdB cassette including the promoter and gRNA scaffold from the vector and open the backbone for assembly of the prepared PCR products by creating specific 3 bp overhangs. SapI also generates overhangs complementary to the plasmid backbone on the 5' end of the first and 3' end of the last PCR product. The second type IIs enzyme creates specific 4 bp overhangs on 3' end of the first, 5' end of the last and each end of all remaining PCR products. The 20 bp of each specific gRNA spacer sequence is split between two primers used to create consecutive parts – e.g. the first half of the first gRNA spacer will be in the 3' end of the first PCR product (also containing the promoter) and the second half of the first gRNA will be in the 5' end of the second PCR product. The 4 bp overhangs created by the second type IIs enzyme are positioned in the middle (positions 9-12) of each gRNA spacer, such that after their ligation a full length (20 bp) gRNA spacer is created. Regarding the fact that the order in which the individual parts are assembled is specified by these 4 bp overhangs in positions 9-12 of each gRNA, bases in these 4 positions should not be identical in any two of the gRNA spacers being assembled. The use of SapI to open the plasmid backbone eliminates the chance that the 4 bp overhang in one of the gRNA spacers will be identical with one of the overhangs in the plasmid backbone, since SapI creates 3 bp overhangs incompatible with the 4 bp overhangs in gRNA spacers. We designed an online tool to assist the user with primer design and gRNA spacers can be analyzed using this tool to avoid repeating sequences in positions 9-12 as well as presence of SapI, Esp3I, BsaI and AarI sites. Although theoretically any number of gRNA spacers could be assembled, the efficiency of this cloning method currently allows for assembly of up to 6 gRNA spacers in a single step. To make constructs with 7 or more spacers, we designed a module C into which additional spacers can be cloned and subsequently assembled with another array of 6 gRNAs in module B by following the regular modular assembly **PROTOCOL 5**. Modifications to the protocol for assembly of 7-12 gRNAs are described below. While cloning into the DIRECT vectors provides the fastest way to build constructs ready for transformation, the modular approach should be used when additional elements (such as a gene targeting donor, TREX2 or GFP expression cassettes) are required in the final vector.



Golden Gate assembly of multiple gRNAs. Primers B and C for one example gRNA spacer are shown on top. The spacer sequence is split between two primers containing the first and last 12 bases of the 20 bp sequence with a 4 bp overlap. Overhangs are underlined. Primers B and C repeat for every new gRNA spacer (up to 6 spacers can be assembled). For example, primer D is equivalent to primer B except the 12 bases specific to another gRNA spacer. The first and last primers (here labeled as A and E, but the number of primers needed depends on the number of gRNA spacers being assembled – up to 14 primers might be needed for assembly of 6 spacers) are universal and vector specific. PCR products containing the promoter and gRNA cassettes are assembled into an array in a single step Golden Gate reaction (in this example using SapI and Esp3I) to yield the final expression cassette (assembly of 4 spacers is shown).