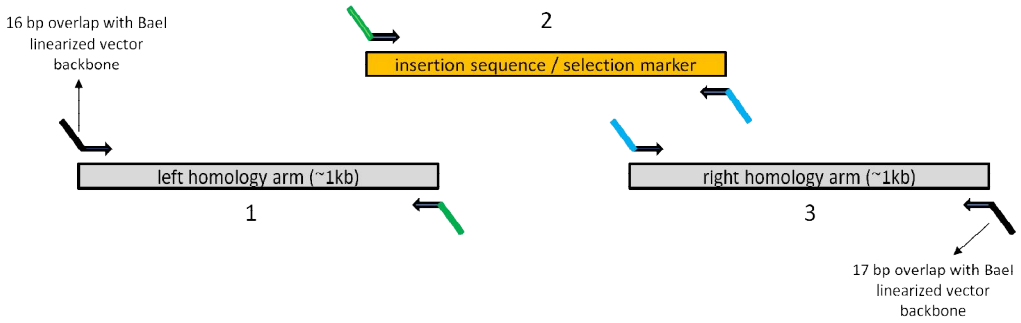
PROTOCOL 4 - DESCRIPTION

Assembly of DNA donor templates for gene targeting into module C vectors

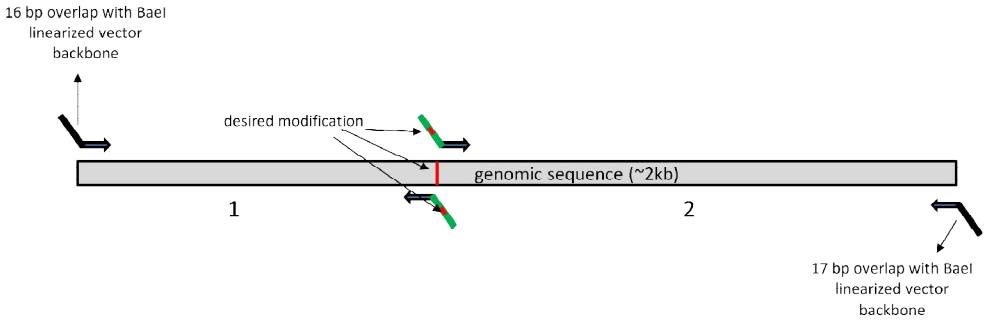
Insertion of DNA donor templates into module C vectors is a simple process which involves PCR and Gibson assembly (Gibson et al., 2009). For targeted insertion of DNA sequences into the genome, the donor template usually consists of three parts, including the left and right homology arms (regions of homology to the targeted genomic site) and the insertion sequence. For allele replacement, a donor consisting of genomic sequence carrying the desired modification is sufficient, but in some cases inclusion of an excisable selection marker cassette may be desired in such donors to aid the selection of Gene Targeting (GT) events. To practice the *in planta* GT method, the donor sequence should be flanked by sequence-specific nuclease (SSN), (TALEN or CRISPR/Cas9) target sites in order to release the donor template from the vector/chromosome. All types of donor templates can be constructed using Gibson assembly as illustrated below. The donor template is amplified by PCR in 2-3 parts with 16 bp overlaps that are assembled directly into the vector backbone. All module C vectors contain a unique BaeI site which is used to open the backbone for Gibson assembly. BaeI is a type IIB restriction enzyme which cleaves the DNA substrate twice to excise its recognition site and creates two distinct 5 bp overhangs. These overhangs have been designed to be incompatible in order to prevent re-ligation of the plasmid backbone without the insert during cloning. Digestion with this single restriction enzyme is sufficient to prepare the vector for Gibson assembly. Since the type IIs enzyme AarI is used to assemble the donor-containing module C into the transformation vector, the donor sequence should be free of AarI sites to prevent miss-assembly. AarI is a rare 7-cutter and the likelihood of finding an AarI site in the sequence of interest is low. However, in the rare cases where one or few AarI sites are present, these can be easily removed in the Gibson assembly process. Another alternative approach to assemble modules containing donors with AarI sites is described in **PROTOCOL 5**. Gibson assembly of donor templates is a cloning step independent of gRNA spacer cloning and therefore two cloning steps are required to build modules containing both gRNA spacer(s) and the GT donor template. gRNA spacer should be cloned first, since the donor sequence may contain type IIs sites that would interfere with gRNA spacer cloning.

Donor template assembly (figure below). In the first approach, the left and right homology arm and the insertion sequence are PCR-amplified. The resulting fragments overlap by 16 bp with each other and with the BaeI digested plasmid backbone as illustrated (pairs of overlapping ends shown as primer extensions are highlighted in the same color). In the second approach, the desired modification is introduced into the genomic sequence by two primers containing the modified sequence in their overlapping extensions. The same approach can be used to remove AarI sites from the sequence. In the *in planta* GT approach, SSN target sites are included in the primer extensions in addition to 16-17 bp of sequence overlapping with plasmid backbone. All fragments (number is not limited to three) are assembled into BaeI digested module C by Gibson assembly.

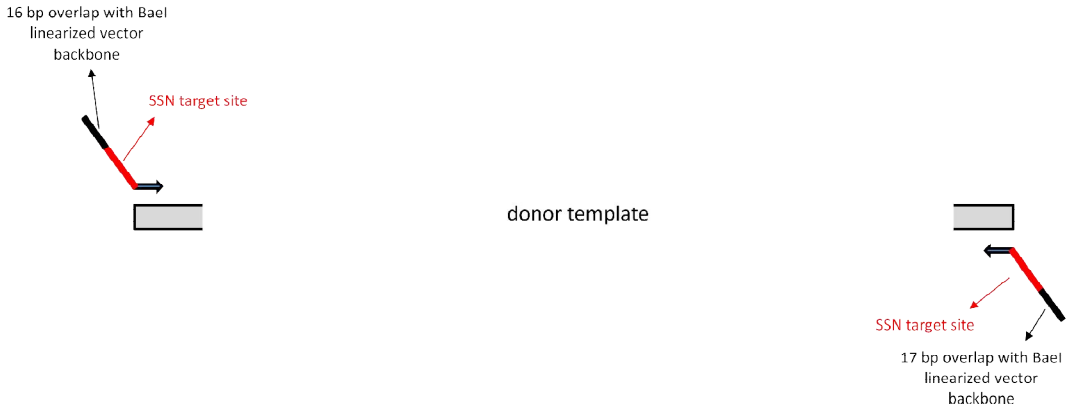
APPROACH 1 – targeted insertion (inclusion of selection marker)



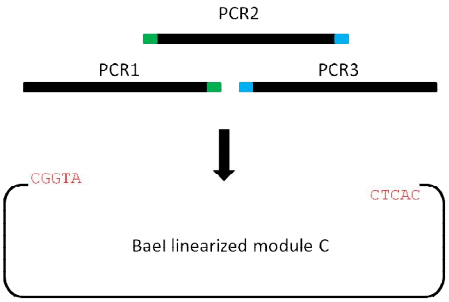
APPROACH 2 – targeted replacement



in planta GT approach



Gibson assembly



REFERENCE

Gibson, D.G., Young, L., Chuang, R.-Y., Venter, J.C., Hutchison, C. A., and Smith, H.O. (2009). Enzymatic assembly of DNA molecules up to several hundred kilobases. Nat. Methods **6**: 343–345.