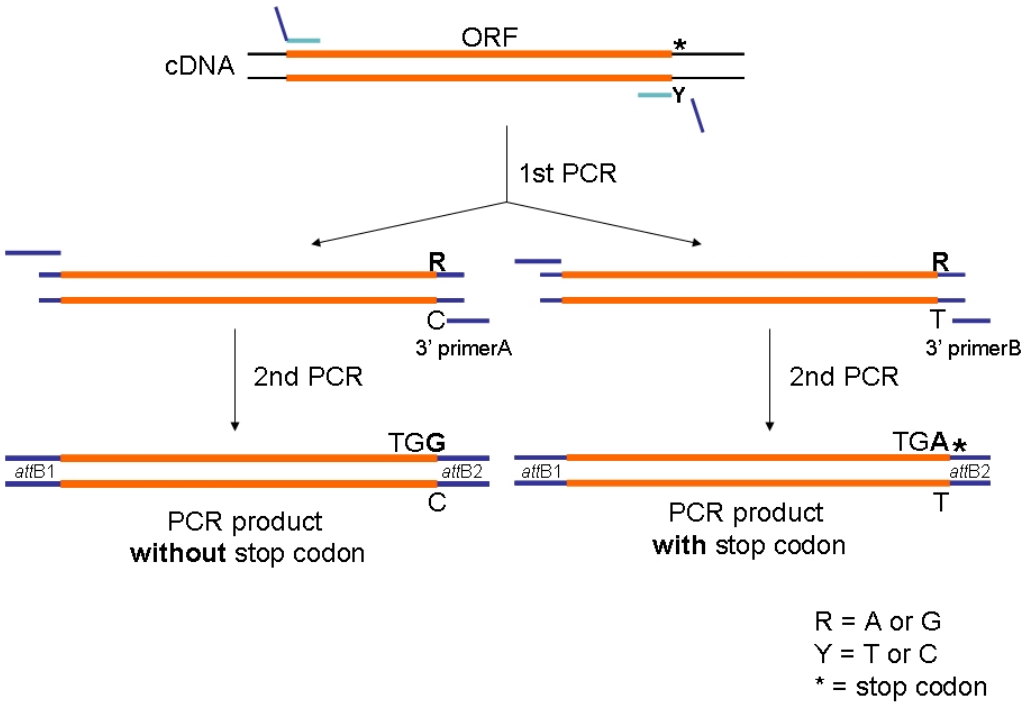
1. Background

Amplification and addition of Gateway recombination sites is done in two consecutive amplification steps. Gene-specific PCR primers amplify the ORF in the first round of PCR, adding minimal overhangs that contain part of the Gateway *att*B-sequences. The second PCR is carried out with a two combinations of "universal Gateway primers" that extend the *att*B-sequences to generate complete recombination sites. Splitting the amplification in two consecutive steps has the advantage of a higher success rate (shorter unspecific overhangs in the first PCR), and the disadvantage of having more cycles (higher error rate induced by PCR enzymes). Having two different 3' primers in the second PCR allows for the amplification of products either lacking or containing a stop codon.



This SOP describes the design of the PCR primers for the two PCR reactions. The primers of the first PCR are mostly gene specific and have only a minimal Gateway-overhang that serves as annealing sites in the second PCR, which is however, carried out in two separate reactions for the open and closed ORFs. There universal primers are used to complete the *att*B cloning sites for the BP-reaction.

Note: The second PCR is carried out separately for the two products of the first PCR, as we have observed the fraction of open and closed ORFs not to be consistently in a 1:1 ratio when the second PCR is carried out in one tube and with the two different 3' primers.

2. Materials

- software for automated design of PCR primers (e.g. Pride [Haas, S., Vingron, M., Poustka, A., Wiemann, S., (1998) Primer design for large scale sequencing. Nucleic Acids Res, 26:3006-12]).
- Sequence of target ORF
- (commercial) supplier of oligos

3. Description of primers

The ORF is tagged in the sequence, starting with the ATG of the translation initiator codon, and ending right upstream of the stop codon. Two types of Entry Vectors shall be generated. One type should retain a stop codon at the original position for expression with N-terminal tags, or for expression of native protein. The second type does not carry the stop codon and thus allows for expression of C-terminal tag-fusions.
Primers are designed to anneal at temperatures around 55°C. Sites are fixed to the ATG and to the last coding codon.
Gateway-specific overhangs are added to the ORF-specific primer sequences:

1. PCR primers for first ORF-specific PCR **(Primer set 1)**
   a. 5' primer: ggC TCC ACC ATg X_n
   b. 3' primer: TGG GTG GAT YCA YYY X_m

- ATGX_n is to indicate gene specific sequence in the primer, starting with the ATG and extending for a variable number of bases (X_n) into the ORF.
- YYYX_m is to indicate gene specific sequence in the primer in reverse complemented format, ending with the last protein coding codon (YYY). The sequence extends for a variable number of bases (X_m) towards the 5' of the ORF.
- The Y in the 3' primer is either C or T, leading to products that either contain a *Bam*HI site (GGATCC), thus having a stop codon (in the sequence context GGATCCGA on the opposite strand), or that have the sequence GGATTC which is not a stop codon on the opposite strand.

The 3' primer is ambiguous in one position. This generates two different PCR products. One has a stop codon, the other has not. The two products can be distinguished after cloning with help of the restriction endonuclease *Bam*HI which cuts if a stop codon is present and does not cut in products containing a stop codon.

2. universal Gateway PCR primers for second PCR **(Primer set 2)**

   a. 5' primer: g ggg ACA AgT TTg TAC AAA AAA gCA ggC TCC ACC ATg
   *att*B1

   b. 3' primerA: ggg gAC CAC TTT gTA CAA gAA AgC Tgg gTg gAT CCA
   *att*B2

   c. 3' primerB: ggg gAC CAC TTT gTA CAA gAA AgC Tgg gTg gAT **TCA**
   *att*B2

The universal Gateway primers in the second PCR anneal to the overhangs that are generated in the products of the first PCR at the conditions of the second PCR. This second PCR is carried out in two independent reactions. The combination of a. and b. generates products lacking a stop codon (codon is TGG), combination a. + c. generates products containing a stop codon (codon TGA).
Primers for first PCR are standard (10 nmol) synthesis without purification

All Primers for second PCR are HPLC or gel purified to avoid n-1 products (frame shift mutations in the products).

The two independent PCRs generate products that either do or do not carry a stop codon. The products can additionally be distinguished with help of the restriction enzyme *Hin*dIII

related SOPs:
ORF-PCR 1 (two step amplification of ORFs based on cDNA)
ORF-PCR 2 (two step amplification of ORFs based on cDNA)

Version	Tracking of changes	Name	Date
2.0	New 3' primers for second PCR to allow for a better distinction between products carrying a stop codon from such that do not.	Wiemann	05/06/24
3.0	The *Hind*III restriction site was replaced by a *Bam*HI restriction site in the 3'-primers of the second PCR. In V2.0 (*Hind*III) the ambiguous position was at position -1 of the second PCR primer, in V3.0 (*Bam*HI) the ambiguous position is at position -3. Upon analysis of PCR products we frequently observed that the 3'-5' exonuclease of the proof reading enzyme had removed the terminal nucleotide of the primer in cases where it did not match the template. In consequence many products of the second PCR were not as expected – some contained stop codons while they should not, and the other way around. By moving the ambiguous position to position -3 has much improved the success rate of specific amplification and cloning, by greatly reducing the fraction of wrong clones.	Bechtel	05/09/28
3.1	New layout	Wiemann	06/01/10