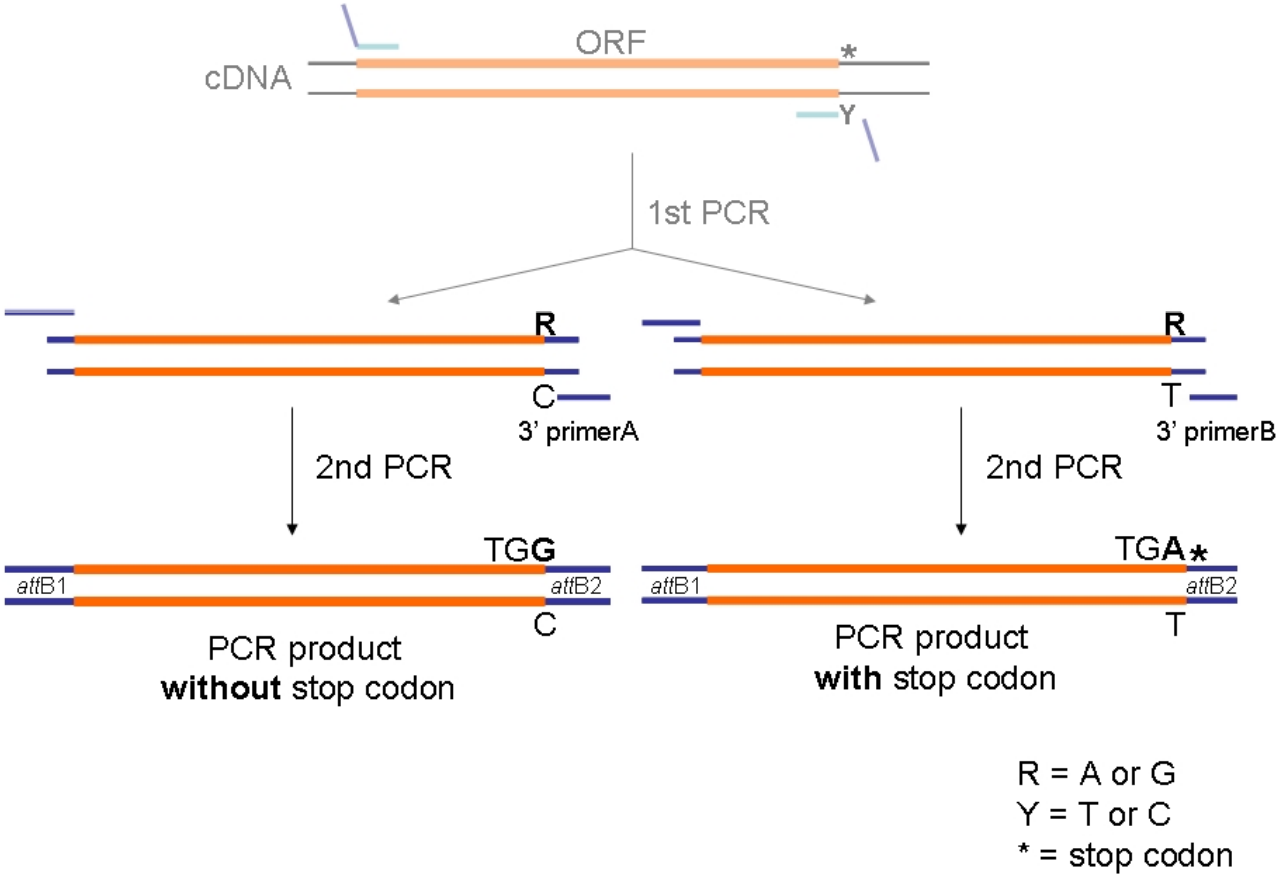
1. Background

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 set up and the procedure of the second PCR that is to extend and complete the Gateway *att*B sites required for recombination cloning. The template for this PCR are generated according to the SOP ORF-PCR 1 (two step amplification of ORFs based on cDNA). Two different sets of primers are used, generating products lacking or containing a stop codon.
The amplification is done in separate reactions because we have observed that ambiguous positions also in the 3' primer of the second PCR do not consistently produce equal numbers of open and closed entry clones in the BP reaction. In consequence more entry clones would be required to screen in order to identify suitable open and closed constructs.

2. Materials

- PCR product from first PCR – SOP ORF-PCR 1 (two step amplification of ORFs based on cDNA)
- enzyme – DNA-dependent DNA-polymerase with proof reading activity, e.g.:
  - Expand High-fidelity (Roche)
  - Phusion polymerase (Finnzymes)
  - KOD polymerase (Novagen)
- 10x reaction buffer
- dNTP solution (each of 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP)
- primer sets 5' primer + 3' primer A, and 5' primer + 3' primer B – see SOP PrimerdesignV3.0.pdf (two-step PCR protocol)
- water
- plastics (Eppendorf tubes, pipette tips, PCR-plates, etc.)
- PCR-machine (e.g. Applied Biosystems)
- micro centrifuge (e.g. Heraeus Biofuge fresco)
- pipettes (0.5-2 µL, 1-20 µL, 10-200 µL, 100-1000 µL)
- equipment for agarose gel-electrophorsis (chamber + power supply, agarose gels (1% in TAE buffer), 50x TAE electrophoresis buffer)
- staining solution (ethidiumbromide)
- unit for image acquisition

3. Methods

PCR set up (single reaction)

This reaction is set up in two independent reactions, where template DNA from the first PCR (contains a W ambiguity) is amplified either with 3' primer a or b. These 3' primers are not ambiguous but contain either an A or T at that position that is ambiguous in the template DNA. In consequence, the products differ in sequence, and in the presence or absence of a stop codon.

2 µL	template DNA (from first PCR)
0.5 µL	5' primer (universal Gateway primer) (10 pmol/µl)
0.5 µL	3' Primers a or b (universal Gateway primers) (10 pmol/µl)
0.5 µL	dNTPs (10mM)
2.5 µL	polymerase buffer
0.5 µL	Enzyme
1.25 µL	DMSO – optional
17.25 µL	H_2O – adjust if DMSO is omitted
25 µL	Final volume

DMSO (up to 5% of final volume) in cases where template is not amplified without (e.g. high GC content)

Thermal cycler program

Expand High fidelity (Roche)		
1.	95°C	2 min
2.	95°C	15 sec
3.	55°C	15 sec
4.	**68°C**	5 min
5.	*Go to step 2 - 5-10 cycles*	
6.	**68°C**	10 min
7.	4°C	∞

Analysis of products:

agarose gel electrophoresis – validation of size, purity, and amount of products

4. next process

SOP BP-reaction (recombination of PCR products into entry vector) – cloning of PCR products into a Gateway-compatible entry vector. Separate BP reactions are carried out for the products of the two PCR reactions.

Version	Tracking of changes	Name	Date
2.0	Two different 3' primers are used in separate PCRs to generate open and closed ORFs for cloning.	Wiemann	05/06/24
3.0	The amount of template DNA was increased to allow for a reduction of the cycle number – reduction of PCR errors	Bechtel	05/09/28
3.1	New layout	Wiemann	06/01/10