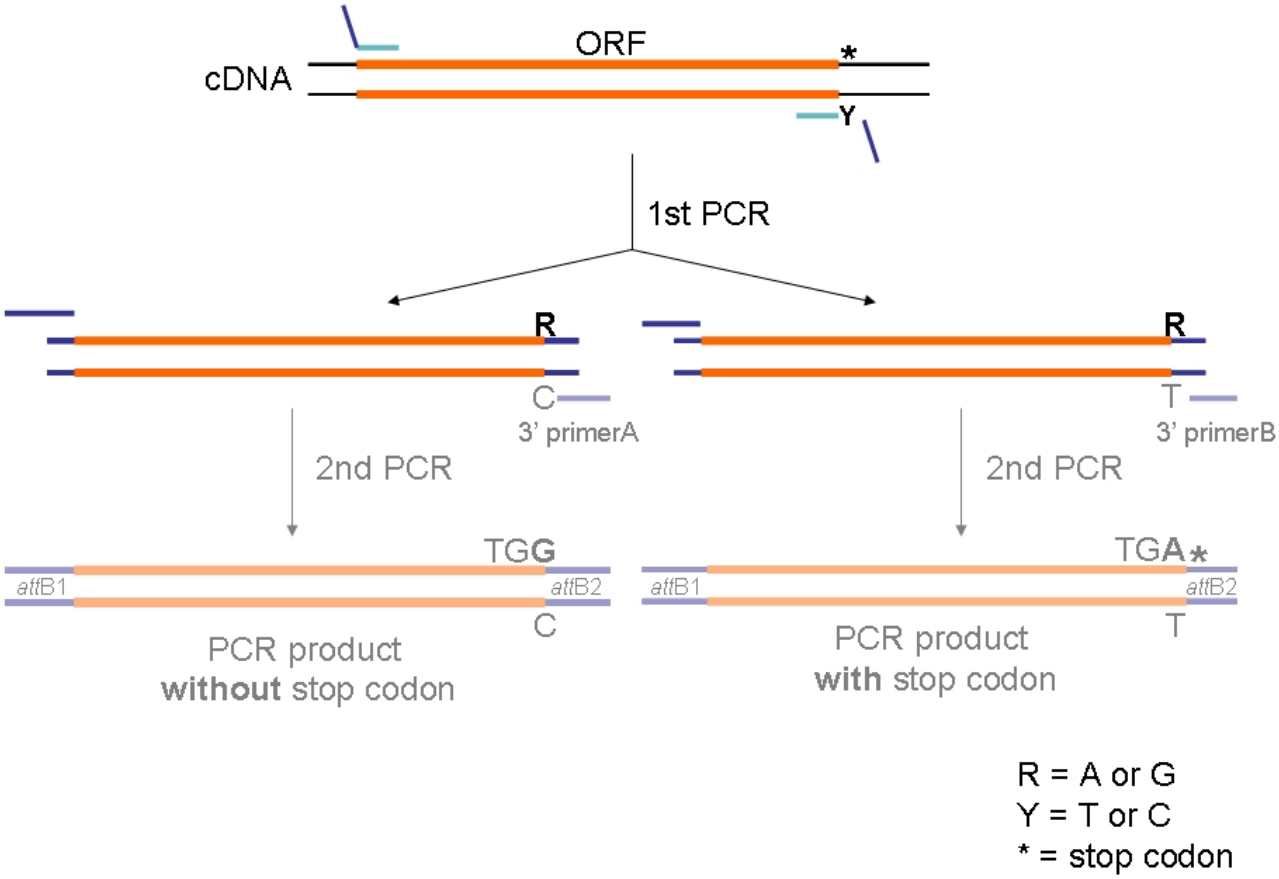
1. Background

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 first PCR that is to amplify the ORF from cloned material. The 3' primer contains an ambiguity (Y = C or T) at the position of the normal stop codon. This generates two types of products (ambiguity code=R), one lacking a stop codon (TGG), the other containing a TGA stop codon.

2. Materials

- plasmid-template DNA (50-100 ng/µL) – purified with standards kits (Qiagen, Macherey&Nagel, Millipore…)
- 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)
- primers – 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)

5 µL	template DNA (50ng – 100ng/µL)
0.5 µL	5' primer (gene specific with 12 bp overhang) (10 pmol/µl)
0.5 µL	3' Primer (gene specific with 12bp overhang) (10 pmol/µl)
0.5 µL	dNTPs (10mM)
2.5 µL	polymerase buffer
0.5 µL	enzyme
1.25 µL	DMSO – optional
14.25 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 ORF-PCR 2 (addition of Gateway *att*B sites) – second PCR to complete the Gateway *att*B sites, prior to cloning of PCR products.

Version	Tracking of changes	Name	Date
2.0	The 3' primer has been modified to contain an ambiguity. This allows for the amplification and cloning of open and closed ORFs.	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