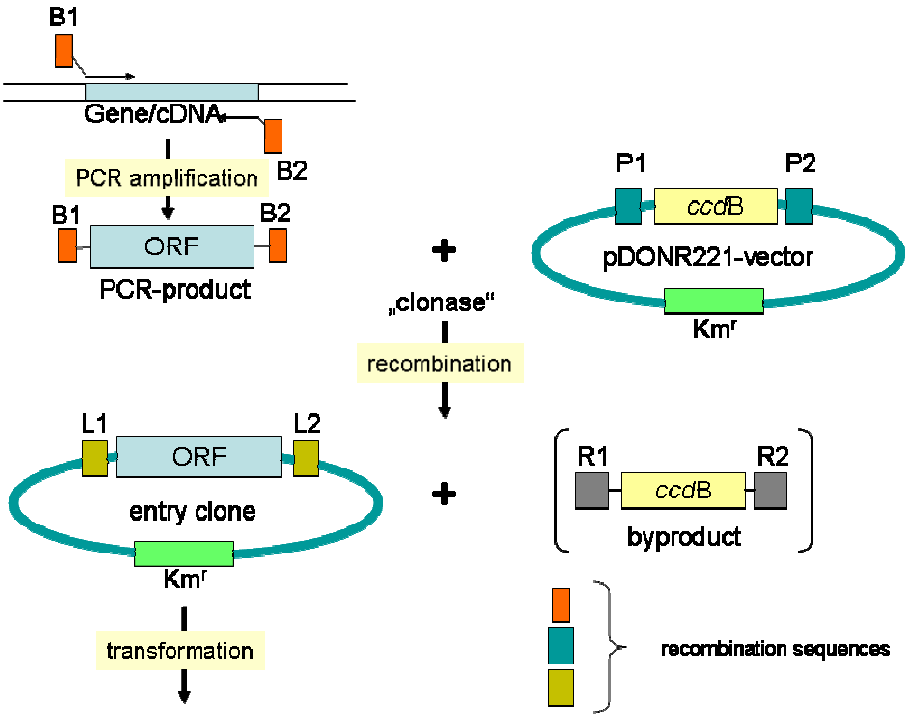
1. Background

This reaction generates bacteria that contain recombinant Gateway-compatible entry plasmid. The PCR-amplified ORFs (SOPs ORF-PCR 1 (two step amplification of ORFs based on cDNA) and ORF-PCR 2 (two step amplification of ORFs based on cDNA)) are recombined into the entry vector with help of a “clonase” of the Gateway cloning system (1). The plasmids are then transformed into bacteria. ORFs are sequence validated, and can then be shuttled into expression vectors, again with the Gateway recombination cloning.



2. Materials

- Strains:

  ***E. coli* strain DB3.1** to propagate Gateway-compatible plasmids (Entry vector and expression vectors). The *gyr*A462 mutation makes this strain resistant fort he *ccd*B-gene product

  Genotype: [F$^-$ *gyr*A462 *end*A1 Δ(*sr*1-*rec*A) *mcr*B *mrr hsd*S20(rB-, mB-) *sup*E44 *ara*14 *gal*K2 *lac*Y1 *pro*A2 *rps*L20(Smr) *xyl*5 Δ*leu mtl*1] von Invitrogen.

  ***E. coli* strain DH10B** to transform products of the BP-reaction. This strain is sensitive to the *ccd*B-gene product. Non-recombinant plasmids are not propagated.

  Genotype: [F$^-$ *mcrA* Δ(*mrr-hsdRMS-mcrBC*) Φ80d*lacZ*ΔM15 Δ*lacX74 endA1 recA1 deoR* Δ(*ara*, *leu*)*7697 araD139 galU galK nupG rpsL*] von Gibco BRL (=Invitrogen).
- Gateway-compatible entry plasmid (pDONR221)
- PCR-products (see SOP ORF-PCR 2 (two step amplification of ORFs based on cDNA))
- Enzyme (BP clonase - Invitrogen)
- BP buffer

  BP –GATEWAY BUFFER

250 mM	Tris/HCl pH 8.0
25 mM	EDTA
125 mM	NaCl
25 mM	Spermidine
5mg/mL	BSA (not acetylated)

- water
- Pellet paint
- 3M NaAc (pH 5.2)
- ethanol (absolut)
- 70 % ethanol (in water)
- LB-medium:

Bacto tryptone	10g
yeast Extract	5g
NaCl	10g
MilliQ-H2O	ad 1000ml

  pH adjusted to 7.2 with NaOH, and autoclaved
  15g/L agar is added prior to autoclaving to make agar plates.
- Antibiotics:

  Kanamycin – stock 50 mg/ml, in water (working concentration 50 µg/ml)
  Agar is cooled to 55°C prior to adding the antibiotic.
- Plastics (Eppendorf tubes, tips, PCR-plates, etc.)
- PCR-maschine
- centrifuge

- electroporation unit (Biorad Gene pulser)
- electroporation kuvettes (0,1 cm electrode distance)
- SOB, LB growth media
- pipettes

3. Methods

BP REACTION

set up of single **reaction** in 0.2 ml Eppendorf tube, multiple reactions in PCR-plate:

BP buffer 5x	2 µL
PCR product	6 µL
entry vector (pDONR221)	1 µL
H_2O	- µL
BP clonase (stored at –80°C)	1 µL
total	10 µL

incubate 2-20h at 25°C (in PCR machine, heat block)
then:

add + 1µL **stop mix** (2µg/µL proteinase K solution)
incubate 10min at 37°C
then:

clean-up to remove salts and proteins

precipitation of DNA (from the Pellet paint precipitation protocol)
add + 2µL Pellet Paint
+ 1/10 Vol 3M NaAC
+ 2 Vol Ethanol 100% p.a.
mix
incubate 2 min at RT
spin 15 min at 13'000 rpm in Eppendorf centrifuge
remove supernatant

70% Ethanol wash
add 100 µL 70% Ethanol
spin 15 min at 13'000 rpm in Eppendorf centrifuge
remove supernatant
air dry pellet

Resuspend pellet in 5 µL H_2O
Use 1µL for **transformation** in DH10B
pick colonies and prep DNA

*Bsr*GI digest of entry-plasmid

Any entry plasmid contains at least two recognition sites for the restriction endonuclease *Bsr*GI that are located in the two att-sites flanking the ORF. This enzyme therefore cuts the ORF-insert from the Gateway entry plasmid and allows for control of insert size as a first measure of QC.

96 format:

1147.5 µL	H2O
157.5 µL	NEB- Puffer 2
15.5 µL	BSA
30 µL	*Bsr*GI

11 µL premix are added to 4 µL of every DNA
incubate for 2 hours at 37°C
products are analyzed by agarose gel electrophoresis

4. next process

positive clones are validated by sequencing

SOP LR-reaction (recombination of ORFs from entry into expression vectors) – cloning of ORFs into a Gateway-compatible expression vector.

Version	Tracking of changes	Name	Date
1.1	New layout	Wiemann	06/01/10

Reference:

1. Hartley, J. L., Temple, G. F., and Brasch, M. A. DNA cloning using in vitro site-specific recombination. Genome Res, *10:* 1788-1795, 2000.