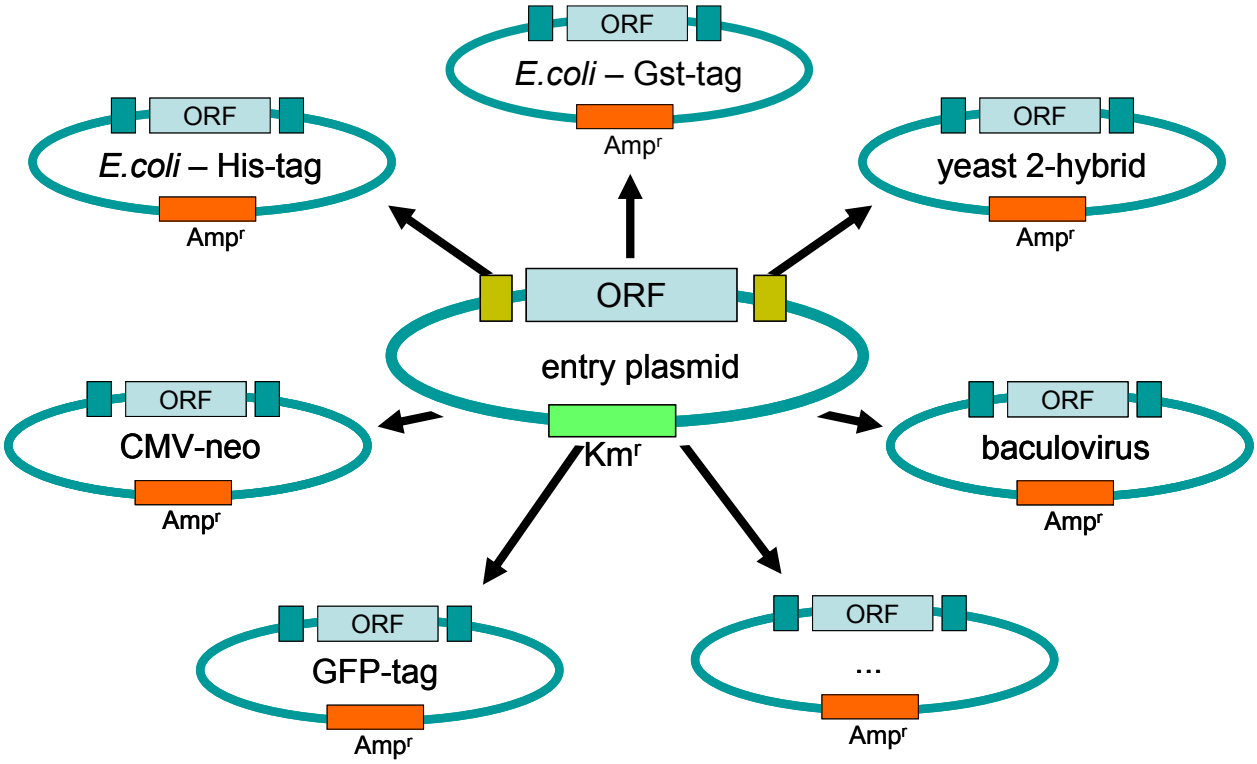
1. Background

This reaction generates bacteria that contain recombinant Gateway-compatible expression plasmids. ORFs are recombined from an entry vector into a range of different expression vectors with help of a “clonase” of the Gateway cloning system (1). The plasmids are then transformed into bacteria.



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 expression plasmid
- Plasmid extracted from entry clone (110-150 ng/µl) (see SOP BP-reaction (recombination of PCR products into entry vector))
- Enzyme (LR clonase - Invitrogen)
- LR buffer

  LR –GATEWAY BUFFER

125 mM	Tris/HCl pH 8,0
25 mM	EDTA
250 mM	NaCl
25 mM	Spermidine
5mg/mL	BSA (nicht acetyliert)

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

Bacto Tryptone	10g
Hefe Extrakt	5g
NaCl	10g
MQ-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:

  Ampicillin – stock 100 mg/ml, in ethanol (working concentration 100 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

LR REACTION

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

	Single reaction
LR Buffer 5x	2 µL
destination vector (150ng/µL)	1 µL
entry plasmid (110 –150ng/µL)	1 µL
H_2O	4.75 µL
Topoisomerase I	0.25 µL
LR clonase	0.5 µL
total	10 µL

set up of Multiple reactions

	(single reaction)	**70 reaction**	**17 reaction**	**5 reaction**
LR Buffer 5x	2 µL	140 µL	34 µL	10 µL
destination vector (150ng/µL)	1 µL	70 µL	17 µL	2,5 µL
entry plasmid (110 –150ng/µL)	1 µL			
H_2O	4.875 µL	271,25 µL	65.875 µL	24.375 µL
Topoisomerase I	0.125 µL	8,75 µL	2.125 µL	0.625 µL
LR clonase	1 µL	70 µL	17 µL	5 µL
total	10 µL			

9 µL of premix is distributed in wells of a 96well plate, 1 µL of entry plasmid is added.

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

*Bsr*GI digest of expression-plasmid

Any expression 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 expression plasmid and allows for control of insert size as a 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

DNA purification and use in experiments (protein expression)

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.