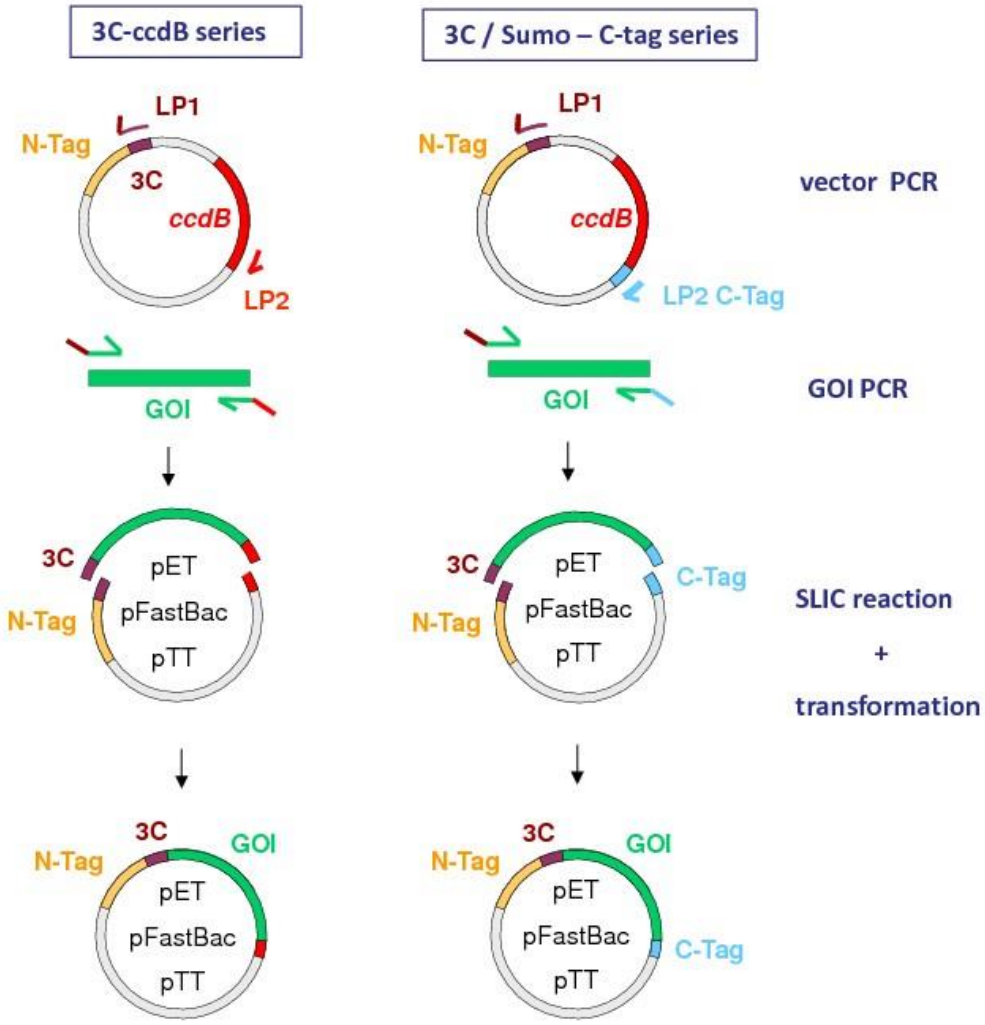
SLIC cloning using the parallel pCoofy vector series

The pCoofy expression vector series was designed for parallel **S**equence and **L**igation **I**ndependent **C**loning [1,2]. We modified expression vectors based on pET, pPICZ, pFastBac and pTT backbones for parallel PCR-based cloning and expression in *E.coli*, *Pichia pastoris*, insect cells and HEK293E cells, respectively. We introduced the toxic *ccdB* gene under control of a strong constitutive promoter that reduces vector background to zero. In addition, the 3' end of *ccdB* functions as a primer binding site common to all vectors. The second shared primer binding site is provided by a PreScission protease cleavage site (3C) located downstream of N-terminal purification and solubility enhancing tags. The gene to be inserted is PCR amplified with primers composed of gene specific sequences plus 20 bp and 25 bp extensions complementary to LP1 and LP2 vector primers. PCR amplification thus generate 20 bp complementary 5' and 3' ends of vector and insert that are joined by homologous recombination during *in vitro* SLIC reaction and transformation in *E.coli* cells.

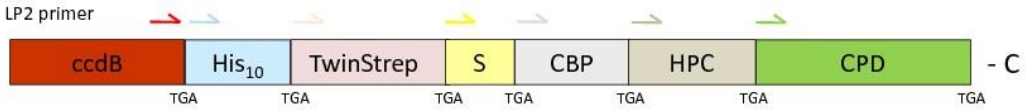


Apart from the parallel 3C-ccdB LP1/LP2 design in the majority of the pCoofy vectors, we have also included N-terminal Sumo tags and C-terminal Multitag cassettes with flexible C-terminal tag insertion depending on the linearization primer used.

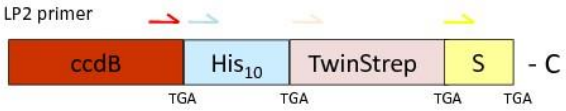
The principle of C-terminal Multi-tag cassettes

When using pCoofys with C-terminal Multi-tag cassettes, the choice of LP2 primer for vector linearization determines the C-terminal tag used. Please note, that these C-terminal tags are not introduced by the primer, but encoded on the vector backbone! All sequences upstream of LP2 will be deleted during PCR linearisation, whereas all tags located downstream will not be translated due to stop codons inbetween. In the course of validation of these C-terminal tags, three series of Multi-tag cassettes have evolved (more details in [2] and Table1).

Multi1



Multi2



Multi3

Vector preparation

1. Propagation/amplification of pCoofys: use the *ccdB* resistant ***E.coli* strain DB3.1**

Note 1: Due to the strong constitutive OmpA promotor driving *ccdB* expression, the "ccdB Survival cells" are NOT suitable for propagating pCoofy vectors. As the *ccdB* gene tends to be inactivated during plasmid propagation even in the resistant strain DB3.1 we recommend frequent "killing controls" using non-permissive E. coli cells.

2. PCR linearization with the appropriate forward LP1 and reverse LP2 primers (see Table 1 and Table 2)

2 µL of vector stock (50 ng/µl)
20 µL 5x GC buffer (NEB)
2 µL Phusion Polymerase (2U/µl, NEB F-30S)
4 µL LP1 primer (10 µM)

4 µL LP2 primer (10 µM)
4 µL dNTPs (10mM, NEB)
70 µL H_2O
Total volume: 100 µL

Transfer 50µl to two PCR tubes, put in a PCR cycler and run the following program:

98°C hold 30sec	98°C - 30sec. 72°C - 90sec. 30 cycles	72°C hold 10min

3. Cleanup: pool both PCR reactions and purify the product using eg High Pure Cleanup Micro Kit (Roche). Elute the DNA in 50 µL autoclaved Millipore H_2O. Measure A_{260}, DNA concentration should be ~ 100 ng/µL.

Note 2: If DNA concentration is significantly lower than 100 ng/µL, the PCR reaction should be repeated and/or optimized.

4. Storage: the linearized vectors can be stored at -20°C ready for SLIC cloning.

Insert preparation

1. Primer Design: Gene specific forward and reverse primer sequences are fused to sequence overhangs of 20-24 nucleotides complementary to the corresponding vector linearization primers LP1 and LP2 as shown in this example (primer sequences are listed in Table 2):

5' GGG CCC CTG GAA CAG AAC TTC CAG 3' Vector **3C - LP1** primer

3' CCC GGG GAC CTT GTC TTG AA 5′ Complementary overhang for LP1 gene primer

5' CGC CAT TAA CCT GAT GTT CTG GGG 3' Vector **ccdB – LP2** primer

3’ GCG GTA ATT GGA CTA CAA GAC CCC 5′ Complementary overhang for LP2 gene primer

Fuse the vector overhang and the gene specific sequence:

3' CCC GGG GAC CTT GTC TTG AA 5′ 3C-LP1 overhang -
ATG GTG AGC AAG GGC GAG GAG CTG 3‘

ATG GTG AGC AAG GGC GAG GAG CTG TTC.. CTC GGC ATG GAC GAG CTC TAC AAG **TGA**
TAC CAC TCG TTC CCG CTC CTC GAC AAG.. GAG CCG TAC CTG CTC GAG ATG TTC ACT

3′GAG CCG TAC CTG CTC GAG ATG TTC ACT

ccdB-LP2 overhang **5' CCC CAG AAC ATC AGG TTA ATG GCG 3‘**

These are the resulting gene linearization primers:

gene LP1 forward primer

5′AA GTT CTG TTC CAG GGG CCC ATG GTG AGC AAG GGC GAG GAG CTG 3‘

gene LP2 reverse primer

5'CCC CAG AAC ATC AGG TTA ATG GCG TCA CTT GTA GAG CTC GTC CAT GCC GAG 3′

Note 3: If no C-terminal tag is included, you need to introduce a stop codon in your LP2 primer. If you forget to include a stop, two more codons from the vector LP2 primer will be translated until the next stop codon is encountered. If you want to include a C-terminal tag, make sure **not** to include the stop codon from your gene. The stop at the end of the C-terminal tag is encoded on the vector backbone.

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2. PCR amplification: the gene of interest is amplified with the properly designed gene LP1 forward and gene LP2 reverse primers.

x µL template*
10 µL 10x reaction buffer
2 µL Phusion Polymerase**
4 µL LP1 primer (10µM)
4 µL LP2 primer (10µM)
4-8 µL dNTPs (10mM, NEB) H_2O
add 100 µL total volume

* the amount of template DNA depends on the DNA used. As a rule of thumb, use 1 µg genomic DNA, 100 ng plasmid DNA or 10-20 ng PCR product.

** In order to generate a specific PCR product, you may have to optimize conditions including polymerases or blends of.

For PCR amplification, take into account insert size and Tm of the primers, Adapt annealing temperature and number of cycles.

98°C hold 30sec	98°C---30 sec. x °C---30 sec. 72°C--- x sec. 30 cycles	72°C hold 10min

Note 4: Analyze the PCR product on a gel and proceed only if you have a distinct specific product of expected size. Non-specific side reactions will fail in subsequent cloning steps! If the PCR failed, optimize conditions. Alternatively, you can order synthetic genes or gene fragments.

Purification of PCR product:

i) If you have amplified a single specific PCR product of proper size and if the antibiotic resistance of the DNA template differs from the target pCoofy vector you do not need to gel purify the PCR product. Proceed as described for the vector PCR.
ii) The pCoofy SLIC cloning procedure does not counter-select template vector. If the template vector antibiotic resistance is identical to the target pCoofy vector, you will mainly obtain template vector background. In this case or in presence of PCR side-products, gel purification of the specific PCR product is mandatory!

SLIC reaction

Ideally, 100 ng of vector is mixed with insert at a molar ratio of 1:3. Practically, we use

1 µL linearised pCoofy (100 ng/µL) + 3 or 4 µl insert (low to medium size) **or**

1 µL linearised pCoofy (100 ng/µL) + 7 µl insert (high size)

1 µL recA protein (NEB MO355S, diluted to 2 µg/mL stock)

1 µL of 10x recA buffer

H_2O add total volume of 10µL

- incubate for 30 min at 37°C, spin down
- pipet directly on 100 µL chemocompetent OmniMAX™ 2 $T1^R$ cells. Cloning efficiency of the competent cells should not be less than 10^7 colonies / µg PUC plasmid DNA.
- incubate 30 min on ice
- heatshock at 42°C for 1 min
- incubate 5 min on ice
- add 200 µL SOC and shake for 1h at 37°C before plating cells on SOB agar
- pick clones and incubate cultures **> 20 hours** (important) for plasmid preparations.

The number of colonies for pET based pCoofys varies from 3-15, for pTT or pFastBac based pCoofys from 10-50 without any background.

Note 5: We have noticed that despite a similar *recA* genotype, background recombination events varied among different cloning strains. The results shown here only apply to OmniMAX™ 2 $T1^R$ cells.

Note 6*:* In our hands, the protocol works without any enzymatic treatment and T4 DNA polymerase treatment of vector and insert. Generating single strand overhangs did not improve cloning efficiency. However, some pCoofy users prefer exonuclease treatment.

References

[1] Li MZ, Elledge SJ: Harnessing homologous recombination in vitro to generate recombinant DNA via SLIC. *Nat Methods* (2007),4 (3):251–256.

[2] Scholz J, Besir H, Strasser C, Suppmann S: A new method to customize protein expression vectors for fast, efficient and background free parallel cloning. *BMC Biotechnology*, 2013,13:12

Table 1: List of pCoofys

Table 2: List of primers

Table 1: pCoofy vector overview

Plasmid Name	Backbone Name	Expression system	Signal peptide N-terminal Tag	Protease Site	C-terminal Tag	LP1 Primer	LP2 Primer	Promoter	Resistance
pCoofy1	pET	E.coli	His6	3C	none	3C	ccdB	T7	Kana
pCoofy2	pET	E.coli	TrxA-His6	3C	none	3C	ccdB	T7	Kana
pCoofy3b	pET	E.coli	GST[1]	3C	none	3C	ccdB	T7	Kana
pCoofy4	pET	E.coli	His6-MBP	3C	none	3C	ccdB	T7	Kana
pCoofy5	pET	E.coli	His6-Sumo1[2]	SenP2[3]	none	Sumo1	ccdB	T7	Kana
pCoofy6	pET	E.coli	His6-Sumo3[2]	SenP2[3]	none	Sumo3	ccdB	T7	Kana
pCoofy7	pET	E.coli	S	3C	none	3C	ccdB	T7	Kana
pCoofy8	pET	E.coli	Halo	3C	none	3C	ccdB	T7	Kana
pCoofy9	pET	E.coli	StrepII (single)[4]	3C	none	StrepII	ccdB	T7	Amp
pCoofy12	pET	E.coli	TwinStrep	3C	none	3C	ccdB	T7	Kana
pCoofy15	pET	E.coli	NusA	3C	none	3C	ccdB	T7	Kana
pCoofy16	pET	E.coli	His10[5]-NusA	3C	none	3C	ccdB	T7	Kana
pCoofy17	pET	E.coli	His10[5]-Sumo3	SenP2[3]	none	Sumo3	ccdB	T7	Kana
pCoofy18	pET	E.coli	His10[5]	3C	none	3C	ccdB	T7	Kana
pCoofy19	pET	E.coli	CBP	3C	none	3C	ccdB	T7	Kana
pCoofy21	pET	E.coli	His10[5]-TwinStrep	3C	none	3C	ccdB	T7	Kana
pCoofy22	pET	E.coli	S	3C	His10	3C	10His	T7	Kana
pCoofy23	pET	E.coli	TrxA	3C	His10	3C	10His	T7	Kana
pCoofy24	pET	E.coli	CBP	3C	His10	3C	10His	T7	Kana
pCoofy25	pET	E.coli	Halo	3C	His10	3C	10His	T7	Kana
pCoofy26	pET	E.coli	NusA	3C	His10	3C	10His	T7	Kana
pCoofy31	pET	E.coli	TwinStrep	3C	His6	3C	6His[6]	T7	Kana
pCoofy33	pET	E.coli	His10[5]	3C	TwinStrep	3C	TwinStrep	T7	Kana
pCoofy34	pET	E.coli	S-TwinStrep	3C	none	3C	ccdB	T7	Kana

pCoofy35	pET	E.coli	MBP	3C	none	3C	ccdB	T7	Kana
pCoofy36	pET	E.coli	S-TwinStrep	3C	His10	3C	10His	T7	Kana
pCoofy37	pET	E.coli	MBP	3C	His6	3C	6His[6]	T7	Kana
pCoofy38	pET	E.coli	TrxA-His10	3C	none	3C	ccdB	T7	Kana
pCoofy49	pET	E.coli	His6-MBP	3C	Multi1: none / 10His / TwinStrep / S /HPC4 / CPD[7]	3C	ccdB / 10His / TwinStrep / S / HPC4 / CPD	T7	Kana
pCoofy52	pET	E.coli	His6-3xAvi	3C	none	3C	ccdB	T7	Kana
pCoofy61	pET	E.coli	His6-eGFP	3C	none	3C	ccdB	T7	Kana
pCoofy27	pFastBac	Baculovirus	His6	3C	none	3C	ccdB	PPH	Amp
pCoofy28b	pFastBac	Baculovirus	GST[1]	3C	none	3C	ccdB	PPH	Amp
pCoofy29	pFastBac	Baculovirus	His6-MBP	3C	none	3C	ccdB	PPH	Amp
pCoofy41	pFastBac	Baculovirus	none or 3C[9]	tagless pFB or 3C	Multi1: none / 10His / TwinStrep / S /HPC4	3C	ccdB / 10His / TwinStrep / S /HPC4	PPH	Amp
pCoofy44	pFastBac	Baculovirus	His10-SumoStar[8]	SumoStar/3C	Multi1: none / 10His / TwinStrep / S /HPC4	SumoStar/3C	ccdB / 10His / TwinStrep / S / HPC4	PPH	Amp
pCoofy51	pFastBac	Baculovirus	TwinStrep	3C	none	3C	ccdB	PPH	Amp
pCoofy59	pFastBac	Baculovirus	TrxA-His6	3C	none	3C	ccdB	PPH	Amp
pCoofy60	pFastBac	Baculovirus	His6-eGFP	3C	none	3C	ccdB	PPH	Amp
pCoofy63	pFastBac	Baculovirus	His6-Smt3Star[8]	Smt3Star	Multi3: none / 10His / TwinStrep / EPEA	Smt3Star	ccdB / 10His / TwinStrep / EPEA	PPH	Amp
pCoofy64	pFastBac	Baculovirus	gp67 - His6Smt3Star[8]	Smt3Star	Multi3: none / 10His / TwinStrep / EPEA	Smt3Star	ccdB / 10His / TwinStrep / EPEA	PPH	Amp
pCoofy11	pIEX	Insect cells transient	His10	3C	none	3C	none	hr5	Amp
pCoofy40	pTT5	Mammalian	none or 3C[9]	tagless pTT5 or 3C	Multi1: none / 10His / TwinStrep / S /HPC4	3C	ccdB / 10His / TwinStrep / S / HPC4	CMV	Amp
pCoofy47	pTT22	Mammalian	SP VEGF - 3C[9]	3C	Multi1: none / 10His / TwinStrep / S /HPC4	3C	ccdB / 10His / TwinStrep / S /HPC4	CMV	Amp

Plasmid Name	Backbone Name	Expression system	Signal peptide N-terminal Tag	Protease Site	C-terminal Tag	LP1 Primer	LP2 Primer	Promoter	Resistance
pCoofy50	pEF-HA	Mammalian	HA-His8-Flag	3C	His10 / none	3C	ccdB / 10His	EF1-alpha	Amp
pCoofy54	PB-T-PAF	Mammalian	TwinStrep	3C	His10 / none	3C	ccdB / 10His	Tet	Amp,Puro
pCoofy55	pTT5	Mammalian	His10-SumoStar[8]	SumoStar/3C	Multi2: none / 10His / TwinStrep / S	SumoStar/3C	ccdB / 10His / TwinStrep / S	CMV	Amp
pCoofy56	pEF-HA	Mammalian	TwinStrep	3C	His10 / none	3C	ccdB / 10His	EF1-alpha	Amp
pCoofy57	pTT22	Mammalian	SP VEGF - His8	3C	Multi1: none / 10His / TwinStrep / S /HPC4	3C	ccdB / 10His / TwinStrep / S /HPC4	CMV	Amp
pCoofy58	pTT22	Mammalian	SP VEGF -TwinStrep	3C	Multi1: none / 10His / TwinStrep / S /HPC4	3C	ccdB / 10His / TwinStrep / S /HPC4	CMV	Amp
pCoofy62	pTT22	Mammalian	SP VEGF-His6Smt3Star[8]	Smt3Star	Multi3: none / 10His / TwinStrep / EPEA	Smt3Star	ccdB / 10His / TwinStrep / EPEA	CMV	Amp
pCoofy65	pTT5	Mammalian	His6-Smt3Star	Smt3Star	Multi3: none / 10His / TwinStrep / EPEA	Smt3Star	ccdB / 10His / TwinStrep / S	CMV	Amp
pCoofy42	pPicZalphaC	Pichia	none (3C)[9]	3C	none	3C	ccdB	AOX1	Zeo

[1]The original pCoofy3 (based on pETM33) and pCoofy28 (based on pFastBac) with His6-GST was discontinued. We have noticed that the combination His-GST substantially decreases protein solubility and the His6-GST tag itself is insoluble.

[2]The first amino acid after the Glycines must **not** be Proline! The protease cannot cleave the protein in that case!

[3]Reverter D and Lima CD: A Basis for SUMO Protease Specificity Provided by Analysis of Human Senp2 and a Senp2-SUMO Complex. *Structure* (2004), 12:1519-1531

[4]The 3C site in pCoofy9 is derived from a different backbone and not identical to the consensus pCoofy LP1 primer

[5]N-His10 has improved IMAC affinity but can lower or abolish expression levels (Keates T *et al*., Expressing the human proteome for affinity proteomics: optimising expression of soluble protein domains and *in vivo* biotinylation. *New Biotechnol.* (2011),29 (5): 515-525)

[6]10His LP2 primer inserts only 6 His

[7]Cysteine-protease-domain (CPD) autocleavage at the C-terminus is induced by inositol hexakisphosphate (InsP6) and can thus not be used in eukaryotic expression systems. CPD autocleavage is a cost-effective alternative to proteases. After self-cleavage only EL remains at the C-terminus. (Shen A *et al*., Simplified, enhanced protein purification using an inducible, autoprocessing enzyme tag *Plos One* (2009),4 (12): e8119 **Please note:** by mistake, the CPD tag of the Multi1 cassette is not followed by a purification tag. This needs to be introduced via primer design.

[8]SumoStar and Smt3Star fusion tags both carry the R64T+R71E mutations described by Liu *et al* (Enhanced protein expression in the baculovirus/insect cell system using engineered SUMO fusions. *Protein Expr Purif.* (2008) 62(1):21-8). However, SumoStar and Smt3Star fusion tags in pCoofys are derived from different parental vectors; therefor the respective LP1 primers for linearization are not identical.

[9]the LP1 primer used adds the 3C sequence GPLEQNFQ to the N-terminus of the protein despite no N-tag only for the parallel cloning purpose; tagless primer can be designed instead

Table 2 Primer for vector amplification and complementary primer extensions for gene of interest (GOI) amplification

LP1 forward vector primer	
3C	5' GGGCCCCTGGAACAGAACTTCCAG 3'
SumoStar	5' TCCTCCAATCTGTTCGCGATGAGCC 3'
Smt3Star	5' TCCGCCGATCTGCTCGCGGTG 3'
Sumo1	5' TCCACCGGTTTGTTCCTGGTAGAC 3'
Sumo3	5' TCCACCGGTCTGCTGCTGGAACAC 3'
StrepII	5' CCCGGGTCCCTGAAAGAGGACTTC 3'
N-tagless pET	5' GGTATATCTCCTTCTCTAGAGGGGAATTGTTATCCGCTC 3'
N-tagless pFastBac	5' GGAATTCCGCGCGCTTCGGACC 3'
N-tagless pTT	5' CGTTTAAACTTGGACCTGGGAGTG 3'
N-tagless PB-T-PAF	5‘ GCTGTACAAACTTGTGATGGCCGC 3‘
LP2 reverse vector primer	

ccdB	5' CGCCATTAACCTGATGTTCTGGGG 3'
10His	5' GAGCATCATCATCATCACCAC 3'
6His	5' GAGCACACCACCACCACCAC 3'
TwinStrep	5' AGCGCTTGGAGCCACCCGCAG 3'
S	5' AAAGAAACCGCTGCTGCTAAATTCG 3'
CBP (calmodulin binding protein)	5' ATGAAGCGGCGGTGGAAGAAAAAC 3'
HPC4 (protein C peptide)	5' GAGGACCAGGTGGACCCCCGG 3'
CPD (cysteine protease domain autocatalytic)	5' GGCAGCGGCAAGATCCTGCAC 3'
EPEA (Capture select C-tag)	5' GAACCGGAAGCGTGAGCGGCCG 3'
LP1 forward gene primer overhang	
3C	5' AAGTTCTGTTCCAGGGGCCC – GOI seq 3′
SumoStar	5' CATCGCGAACAGATTGGAGGA – GOI seq 3′
Smt3Star	5' CACCGCGAGCAGATCGGCGGA – GOI seq 3′
Sumo1	5' GTCTACCAGGAACAAACCGGTGGA – GOI seq 3′
Sumo3	5' GTGTTCCAGCAGCAGACCGGTGGA – GOI seq 3′
StrepII	5' GAAGTCCTCTTTCAGGGACCCGGG – GOI seq 3′
N-tagless pET	5' GAGCGGATAACAATTCCCCTCTAGAGAAGGAGATATACC – GOI seq 3′
N-tagless pFastBac	5' GGTCCGAAGCGCGCGGAATTCC – GOI seq 3′
N-tagless pTT	5' CACTCCCAGGTCCAAGTTTAAACG – GOI seq 3′
LP2 reverse gene primer overhang	
ccdB	5' CCCCAGAACATCAGGTTAATGGCG - Stop – GOI seq 3′
10His	5' GTGGTGATGATGATGATGCTC – GOI seq 3′
TwinStrep	5' CTGCGGGTGGCTCCAAGCGCT – GOI seq 3′
S	5' AGCAGCAGCGGTTTCTTT – GOI seq 3′
CBP (calmodulin binding protein)	5' CTTCCACCGCCGCTTCATC – GOI seq 3′
HPC4 (protein C peptide)	5' GGGGTCCACCTGGTCCTC – GOI seq 3′
CPD (cysteine protease domain autocatalytic)	5' CAGGATCTTGCCGCTGCC – GOI seq 3′
EPEA (Capture select C-tag)	5' TCACGCTTCCGGTTC – GOI seq 3′