Protein Production & Assays

For research use only

Protocol

Direct transfer cloning into StarGate vectors

Generation of expression vectors by conventional cloning

1 DESCRIPTION

StarGate has been developed for the rapid systematic screen of the optimal expression and purification system for a given gene of interest (GOI). It offers a “two-step-cloning” procedure for rapid and highly efficient subcloning of an arbitrary gene. In the first step, the GOI is cloned in a donor vector from which it can be subcloned by a standardized procedure into a variety of acceptor vectors. Acceptor vectors provide different genetic surroundings without the need for sequencing each individual vector. The generated final expression vector is placed into the respective host.

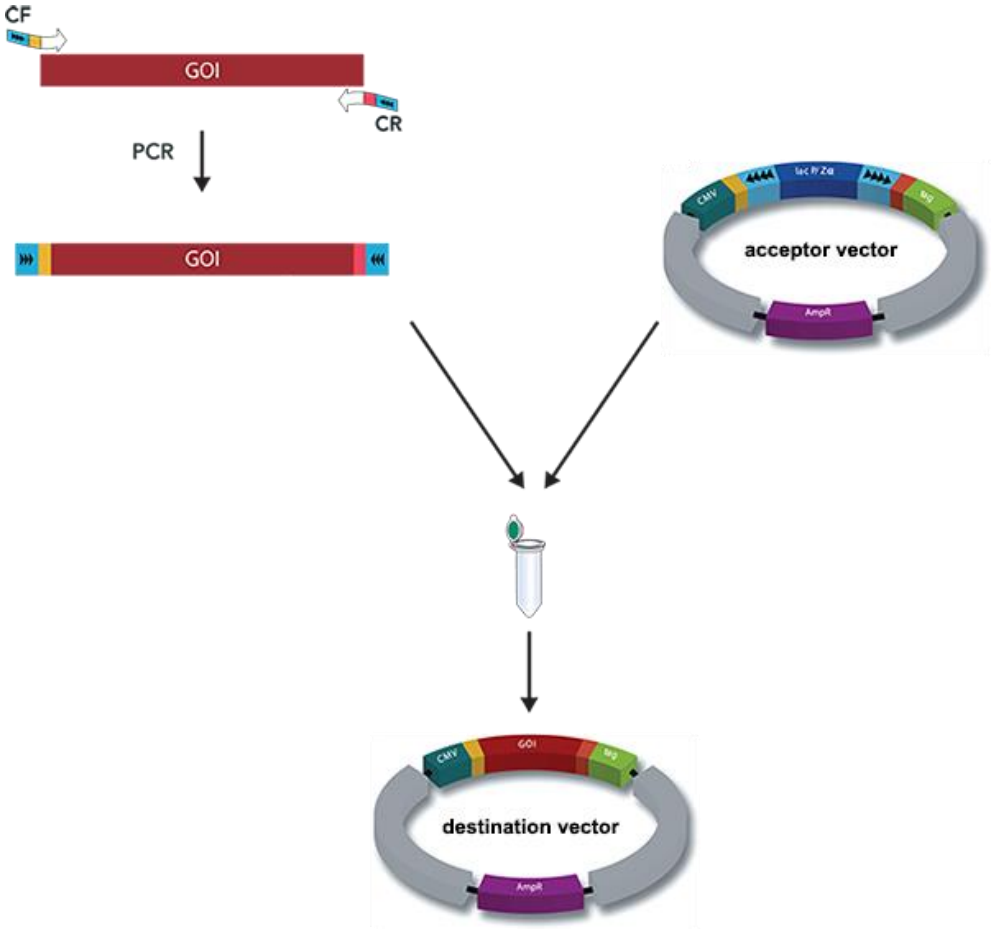
If the optimal expression and purification system is already known, a polymerase chain reaction (PCR) product containing the gene of interest can be directly inserted into the appropriate acceptor vector without the need for prior generation of a donor vector. This “direct transfer cloning” approach using appropriately designed PCR primers is described in this protocol.

PR82-0008, May 2022

2 GENERAL INFORMATION AND NECESSARY COMPONENTS

2.1 Direct transfer cloning procedure

First, the PCR fragment is generated using the respective forward (CF) and reverse (CR) primer extending the GOI with the corresponding integration sites. This PCR product is then integrated into the appropriate acceptor vector resulting in the final expression construct, called destination vector. The formation of the correct destination vector is monitored via blue-white screening on LB-Agar plates containing 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside(X-Gal). The validated destination vector can be directly used for transformation or transfection of the expression host.

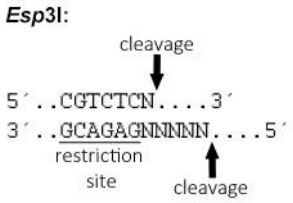


2.2 Primer design for amplification of GOI

In case of using a proofreading polymerase, which is highly recommended (e.g., *Pfu*), 3'-phosphorothioate protected primers should be used. Otherwise, proof reading activity may degrade the primers from the 3'-end during PCR, impairing annealing and consequently reduces the yield of PCR product.

Essential parameters for PCR optimization are the annealing temperature, the duration of synthesis and the template concentration. Initial hybridizing regions of primers (marked with | in the example 2.3) should have a theoretical melting temperature between 60 °C and 63 °C. The primer melting temperatures can be derived by adding the single base melting temperatures of consecutive bases using 4 °C for each GC pairing and 2 °C for each AT pairing (and 1 °C for each GT pairing). The annealing temperature should be chosen at least 5 °C below the melting temperature of each primer. The number of cycles should be kept as low as possible to minimize base substitutions.

The integration site that needs to be attached by PCR to the 3'- and 5'-end of the GOI consists of an *Esp*3I recognition site. *Esp*3I is a type IIS restriction enzyme that cleaves the DNA in double strand outside the recognition site (4 bases, here NNNN, see scheme below). Thereby, digestion with only one single enzyme can generate two different independent sticky ends with 4-base 5'-overhangs allowing directional cloning. In addition, after digestion reaction the recognition sequence is removed completely, and the encoded amino acid sequence is not affected by remaining restriction enzyme sites. Hence, even the expression of authentic proteins is possible.



2.3 Example

If the subsequent sequence would represent a GOI (start and stop codon are left out)

```
5′-TTGACCTGCAACAGCTGCATAGCC-3′
3′-AACTGGACGTTGTCGACGTATCGG-5′
   LeuThrCysAsnSerCysIleAla
```

then the following primers have to be designed for PCR to equip GOI with the needed sites:

```
   (CF: forward primer)
5′-AGCGCGTCTCCAATGTTGACCTGCAACAGCTGCAT-3′
                ||||||||||||||||||||
               5′-TTGACCTGCAACAGCTGCATAGCC-3′
               3′-AACTGGACGTTGTCGACGTATCGG-5′
                        ||||||||||||||||||||
                     3′-GACGTTGTCGACGTATCGGCCCTCCTCTGCGCGA-5′
                          (CR: reverse primer)
```

5’ end of the CF primer (forward primer) is elongated by the AATG combinatorial site (italic and bold) and the *Esp*3I recognition site (underlined) and

5’ end of the CR primer (reverse primer) is elongated by the reverse complement (CCCT) of the downstream combinatorial site GGGA and again with the *Esp*3I recognition site (underlined).

The resulting PCR product then has the following structure:

```
5′-AGCGCGTCTCCAATGTTGACCTGCAACAGCTGCATAGCCGGGAGGAGACGCGCT-3′
3′-TCGCGCAGAGGTTACAACTGGACGTTGTCGACGTATCGGCCCTCCTCTGCGCGA-5′
              MetLeuThrCysAsnSerCysIleAlaGly
```

2.4 Necessary components

IBA Lifesciences offers acceptor vectors for protein expression in *E. coli*, mammalian cells, insect cells or yeast with Strep-tag®II, Twin-Strep-tag®, GST-tag, Flag-tag and His-tag. An overview of available acceptor vectors is given in section 4. For propagation of plasmid DNA chemical competent *E. coli* TOP10 cells (5-1600-020) are provided as well. Necessary enzymes, buffers and primers are listed in the following tables and can be obtained from other suppliers.

Necessary enzymes and buffers	
dNTP Mix	Containing dATP, dTTP, dGTP, dCTP
Pfu DNA Polymerase and supplied buffer	
T4 DNA Ligase	Concentration 1 U/µl
DTT	Often provided with T4 DNA Ligase
ATP	Often provided with T4 DNA Ligase
Esp3I restriction enzyme and supplied buffer	Concentration 10 U/µl. The enzyme must be active at 37 °C. Do not use the isoschizomer *BsmB*I

Sequencing Primers (HPLC purified)	
5’-GAGTTATTTTACCACTCCCT-3’	Forward sequencing primer for pASG-IBA and pASK-IBA vectors (*E. coli* vectors)
5’-CGCAGTAGCGGTAAACG-3’	Reverse sequencing primer for pASG-IBA and pASK-IBA vectors (*E. coli* vectors)
5’-GAGAACCCACTGCTTACTGGC-3’	Forward sequencing primer for pESG-IBA, pCSG-IBA, and pDSG-IBA (mammalian vectors)
5’-TAGAAGGCACAGTCGAGG-3’	Reverse sequencing primer for pESG-IBA, pCSG-IBA, and pDSG-IBA vectors (mammalian vectors)
5’-CAATATCATATAGAAGTCATCGA-3’	Forward sequencing primer for pYSG-IBA vector (yeast vectors)
5’-GCAGCTACCACATTGGCATTGGC-3’	Reverse sequencing primer for pYSG-IBA vector (yeast vectors)
5’-TAACCATCTCGCAAATAAATAAG-3’	Forward sequencing primer for pLSG-IBA vector (insect cells)
5’-CAACGCACAGAATCTAGCGC-3’	Reverse sequencing primer for pLSG-IBA vector (insect cells)

3 PROTOCOL

3.1 PCR amplification of the GOI with *Pfu* polymerase

The following protocol is based on standard protocols for *Pfu* polymerase PCR.

3.1.1 Mix the following reagents in a PCR tube and a total volume of 50 µl:

Concentration	Reagent
200 µM	dNTP
0.1-0.5 µM	forward primer
0.1-0.5 µM	reverse primer
5 µl	10x buffer (supplier)
20-200 pg/µl plasmid DNA **Or** 0.1-1 ng/µl cDNA library	template DNA
2.5 U	*Pfu* polymerase*
Ad 50 µl	distilled H_2O

*Depending on the recommendations of the manufacturer. *Pfu* can also be added after the initial denaturation step

3.1.2 Use a heated lid if available. Alternatively, overlay the sample with mineral oil.

3.1.3 Start temperature cycling:

Temperature	Time	Number of cycles	PCR step
94 °C	180 s	1	Initial denaturation
94 °C	30 s	15-20 cycles for plasmid DNA or 30-40 cycles for cDNA library	Denaturation
55-65 °C	30-60 s		Annealing
72 °C	30-240 s		DNA synthesis
60-72 °C	300 s	1	Terminal extension
4 °C	∞	1	Storage

3.1.4 Quantify the PCR fragment by comparing the band intensity with a DNA standard. Apply two different amounts of PCR product in separate lanes to find a band of equal intensity in the DNA standard, which has to be applied to the same gel. Alternatively, quantify via NanoDrop measurement.

3.1.5 Isolate the PCR product. If multiple bands are visible after gel electrophoresis, only isolate the PCR product of the expected size from the gel. Use of a DNA purification kit to extract the PCR fragment is recommended.

3.2 GOI transfer into the acceptor vector

When working with *E. coli* cells it is important that they are not vortexed, as shearing forces can damage the cells.

3.2.1 Prepare a DTT/ATP Mix:
250 mM DTT
12.5 mM ATP
The mix can be stored in small aliquots at -20 °C.

3.2.2 Mix the following reagents for insertion of the GOI into the chosen acceptor vector:

Amount	Reagent
7.5 µl	Accpetor vector (5 ng, dilute in distilled water)
2.5 µl	Buffer supplied with *Esp*3I
12.5 µl	PCR product (2 nM, from section 3.1)
1 µl	DTT/ATP Mix
1 µl	T4 DNA Ligase (1 U/µl)
0.5 µl	*Esp*3I (10 U/µl)

3.2.3 Close the reaction vessel thoroughly to avoid evaporation. Mix gently and incubate at 30 °C for 1 h.

3.2.4 Thaw a vial of competent *E. coli* cells on ice.

3.2.5 After incubation, pipet off an aliquot of 10 µl from the reaction mixture from step 2 and add it to the thawed competent *E. coli* cells. Store residual reaction mixture (15 µl) at 2-8 °C for backup.

3.2.6 Mix gently and incubate for 30 min on ice.

3.2.7 Mix gently and incubate for 5 min at 37 °C.

3.2.8 Mix gently and incubate for 2-5 min on ice.

3.2.9 Mix 10 µl of the mix with 90 µl LB medium. Plate the diluted mix and the remaining 100 µl on LB agar containing 100 mg/l ampicillin and 50 mg/l X-gal.

3.2.10 Incubate plates over night at 37 °C (upside down).

3.3 Destination vector identification

3.3.1 Pick 3 white colonies and perform DNA mini preparation.

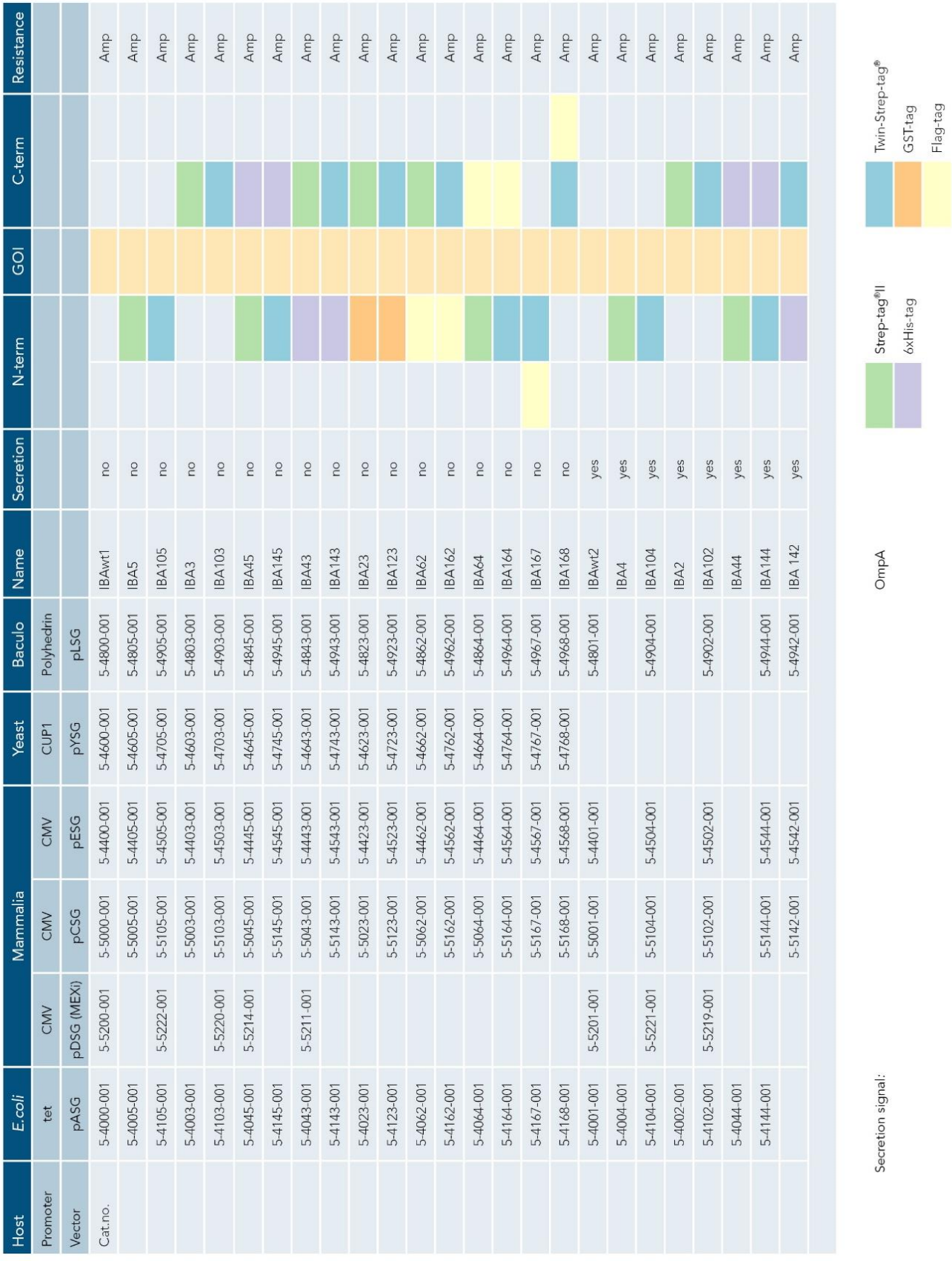
3.3.2 pASG-IBA, pESG-IBA, pCSG-IBA, pDSG-IBA and pYSG-IBA have *Xba*I/*Hind*III restriction sites that flank the expression cassette and may be used for confirmation of GOI integration. Due to an additional *Hind*III site downstream to GOI, an additional fragment of 456 bp will be generated after *Xba*I/*Hind*III cleavage of pLSG-IBA vectors. For exact calculation of expected restriction fragment length please refer to the appropriate acceptor vector data sheet.

3.3.3 Validate the correct sequence of your GOI by sequencing. Sequences for primers specific for the acceptor vector are listed in section 2.4.

3.3.4 Use the destination vector to transform the corresponding host.

4 ACCEPTOR VECTOR COLLECTION OVERVIEW

The following list provides information about the expression host, used promoter, available secretion signal and cloning site including N- or C-terminal tag as well as the cat. no. of each expression vector. The vector name (e.g., pASG-IBA5) comprises the expression system (pASG = *E. coli*/tet) and the expression cassette (affinity-tag/position/secretion signal, IBA5 = Strep-tag®II/N-term/no secretion signal).



Check our Downloads page

www.iba-lifesciences.com/download-area.html

for the latest version of this manual.

Info on warranty / licensing and trademarks available at:

www.iba-lifesciences.com/patents-licenses-trademarks.html

If you have any questions, please contact

strep-tag@iba-lifesciences.com

We are here to help!