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Solutions
For Life Sciences

StarGate®

The new dimension of combinatorial cloning

Instruction manual

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www.stargate.com

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Content

This manual can be downloaded from **www.iba-lifesciences.com/technical-support.html**.

1 Introduction

Efficient procedures for functional expression, purification, detection, and immobilization or separation of recombinant proteins – possibly in complex with cognate macromolecules – are of key importance in modern protein science. Many tools like various expression hosts (bacteria, yeast, insect and mammalian cells), promoters, affinity or fluorescent tags are currently available to fulfil these tasks. Due to the heterogenic nature of proteins, however, it is impossible to predict which combination of these tools will perform best in a certain situation. Therefore many have to be tried in order to identify an optimal solution.

To systemize and accelerate this initial search, which is crucial for successful subsequent proteomic research, IBA has developed the StarGate® system. StarGate® offers a “two-step-cloning” procedure for rapid and highly efficient subcloning of an arbitrary gene. In a first step the gene is cloned into pENTRY-IBA to obtain the so called Donor Vector. In a second step the gene can be easily and in parallel transferred from the Donor Vector into Acceptor Vectors, which provide different genetic surroundings. The final expression vector is called Destination Vector and is placed into the respective host.

In this manual we describe the generation of Destination Vectors in order to express one or more proteins from a single vector.

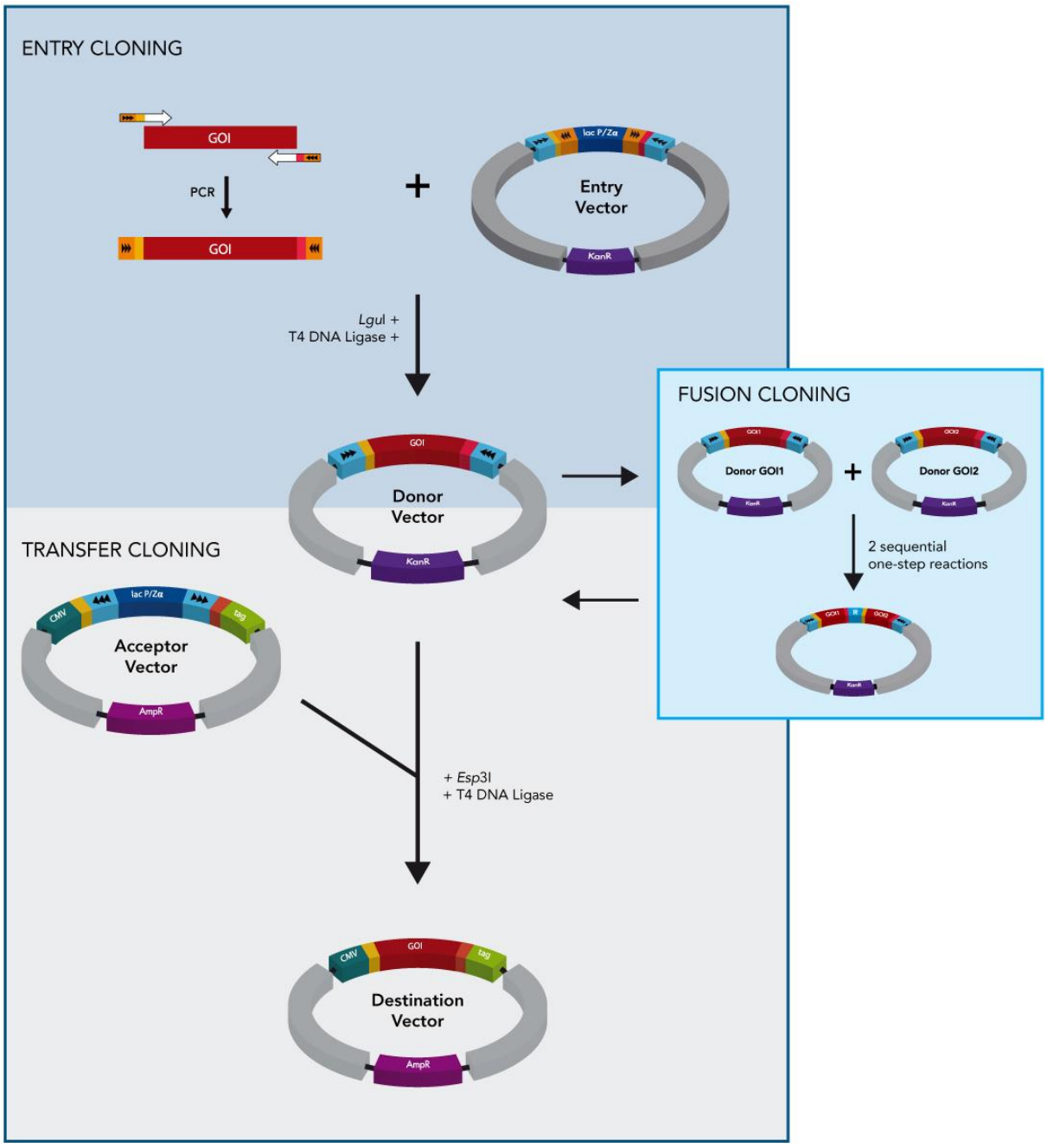
Alternatively, the gene of interest can be directly cloned into the Acceptor Vectors in cases where the optimal expression system is known (use **StarGate® - Direct Transfer Cloning Manual**).

Key advantages of StarGate® are

- minimal extra modification of the gene of interest due to short combinatorial sites
- inherent high level cloning efficiency due to a directed reaction (no equilibrium)

2 StarGate® procedure

2.1 Workflow (schematic view)



Step 1: Donor Vector generation

In the first step, the gene of interest (GOI) is extended by PCR at both ends with the corresponding integration sites (primers may be conveniently designed by the free software "StarPrimer D'Signer" available at www.iba-lifesciences.com). Subsequently, the PCR product is inserted into the Entry Vector by a simple one-tube reaction. See Chapter 2.2/ 3.1/4.1 for detailed description.

Optional intermediate step: Fusion Cloning

StarGate® fusion cloning is an additional step between the Entry reaction and the Transfer reaction. It allows easy and fast linkage of two genes of interest (GOI-1 and GOI-2) present in separate Donor Vectors via an intergenic region (IR), that can be a linker, Shine Dalgarno (SD) or IRES element, by performing two sequential StarGate® subcloning reactions. Please refer to chapters 2.3/3.2/4.2.

Step 2: Destination Vector generation (Transfer Cloning)

After sequence confirmation the Donor Vector serves as basis for subcloning of the GOI into a multitude of Acceptor Vectors by a second simple one-tube reaction. Each Acceptor Vector provides a different genetic surrounding like host specific promoters and different purification tags. The obtained Destination Vectors are finally transferred into the desired expression host. Ultimately, optimal expression and purification conditions of a given GOI can be systematically screened. See Chapter 2.4/3.3/4.3 for detailed description.

2.2 Step 1: Donor Vector generation

First, the gene of interest (GOI) has to be equipped with the integration sites, consisting of a *Lgu*I recognition site and a 4 base comprising combinatorial site at both termini. They are important for oriented insertion of the PCR fragment into pENTRY-IBA51 (Figure 1).

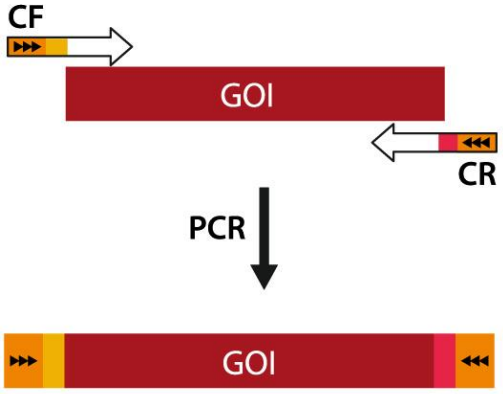


Fig. 1
The forward primer (CF) starts with a sequence containing the *Lgu*I recognition site (GCTCTTC; orange with arrows indicating its orientation) followed by the downstream AATG combinatorial site. Here the start codon ATG is already included. The primer sequence continues with nucleotides complementary to the antisense strand of the GOI.
The reverse primer (CR) equally starts with a sequence containing the *Lgu*I recognition site (GCTCTTC) followed by TCCC (the reverse complement of the downstream combinatorial site GGGA), which again is directly followed by a sequence which is reverse complementary to the 3'-end of the GOI.

Example:

The GOI has the following sequence (leave out the Met start codon and the stop codon):

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

Appropriate primers have to be designed (use StarPrimer D'Signer or refer to 4.1.1.1) so that the resulting PCR product will additionally include the combinatorial sites (bold and italic), the *Lgu*I recognition site (underlined) and 4 additional bases for efficient restriction enzyme activity. In this example, the resulting PCR product has the following sequence:

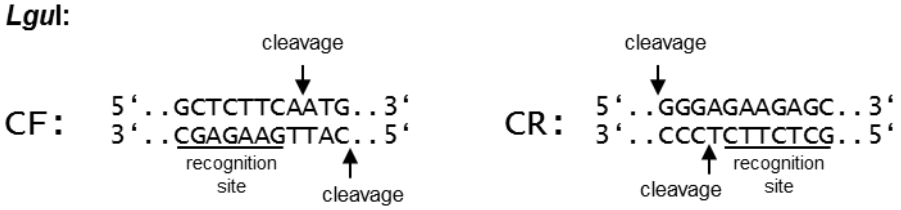
```
5′-AGCGGCTCTTC***AATG***TTGACCTGCAACAGCTGCATAGCC***GGGA***GAAGAGCCGCT-3′
3′-TCGCCGAGAAG***TTAC***AACTGGACGTTGTCGACGTATCGG***CCCT***CTTCTCGGCGA-5′
          *Met*LeuThrCysAsnSerCysIleAla*Gly*
```

The Met start codon is reconstituted by the upstream combinatorial site and the stop codon is replaced by a glycin "GGG" codon included in the downstream combinatorial site to allow C-terminal fusions of the GOI.

Important notes

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

***Lgu*I:**



Tip for proper PCR

- Use a proof reading DNA polymerase like *Pfu* (Thermo Scientific) that minimizes the risk of mutations.
- Use 3' phosphorothioate (PTO) protected primers in case of using a proof reading DNA polymerase.

The obtained PCR product is inserted into the Entry Vector pENTRY-IBA51 which thereby converts into a Donor Vector (Figure 2).

This is achieved by mixing the Entry Vector with the PCR product, the restriction enzyme *Lgu*I and T4 DNA Ligase. The resulting mixture is incubated for one hour.

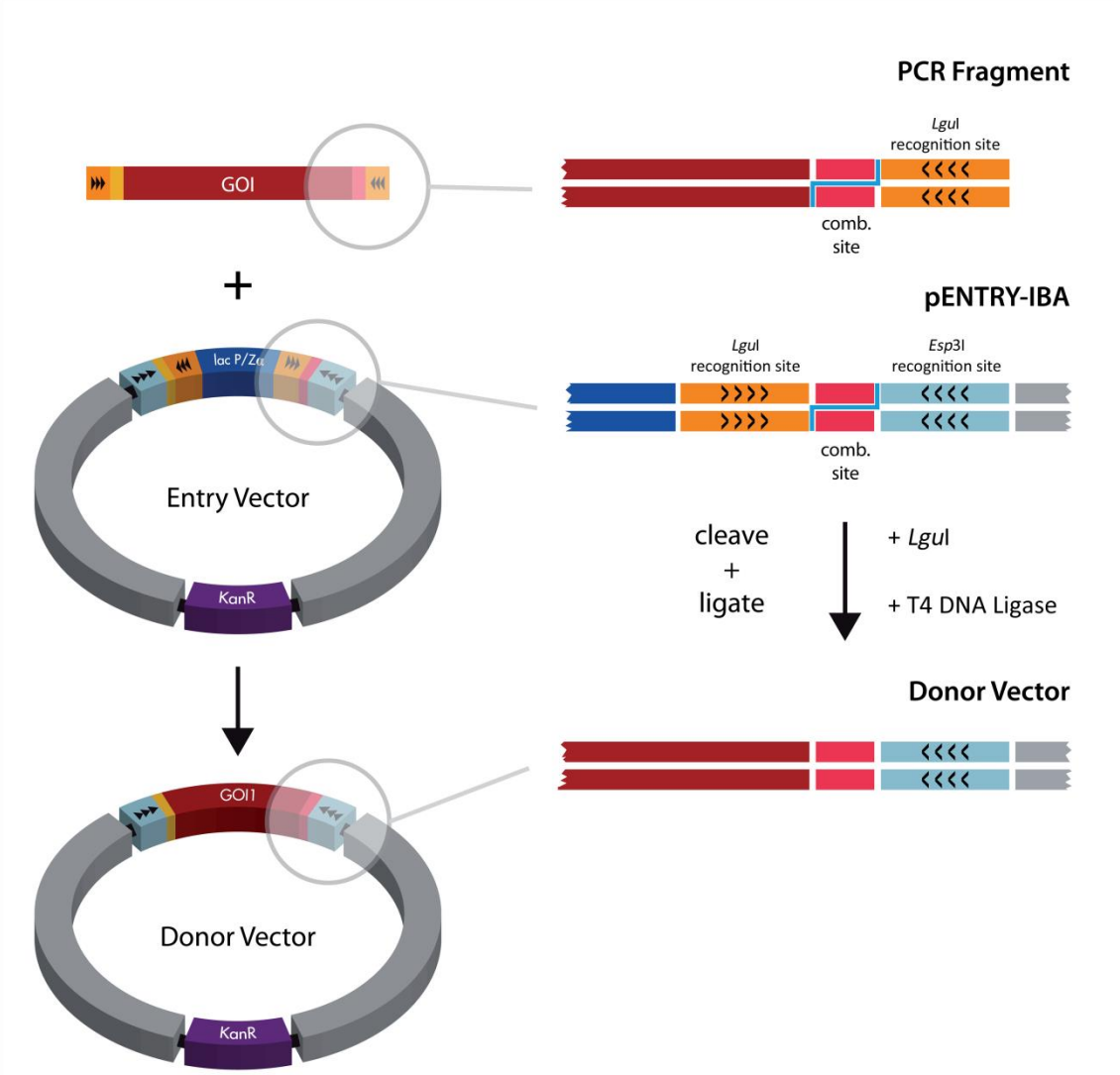


Fig. 2
Insertion of GOI into the Entry Vector pENTRY-IBA51 results in a Donor Vector.

In a straight forward one-step reaction, *Lgu*I restriction enzyme cleaves the PCR product and the Entry Vector. Unlike other restriction enzymes LguI cleaves next to its recognition site, thereby creating overhangs which are named combinatorial sites. Then, T4 DNA ligase generates the final Donor Vector. Thus, the PCR product and the Entry Vector are recombined at the combinatorial sites (red and yellow), accompanied by loss of the *Lgu*I recognition sites (orange). This makes the recombination reaction unidirectional and thereby highly efficient.
The same combinatorial sites in the resulting Donor Vector are now flanked by *Esp*3I recognition sites. This enables a highly efficient and specific GOI transfer process into correspondingly designed Acceptor Vectors.

Insertion of PCR product/GOI into the Donor Vector can be checked by restriction analysis. As PCR, however, may lead to mutations and to improper product ends, it is recommended to confirm GOI and flanking sequences by sequencing using ENTRY-Primer-for2 and/or ENTRY-Primer-rev.

2.3 Fusion of two or more GOI's via Fusion Cloning

Two or more genes that are already cloned into separate Donor Vectors can be connected by an intergenic region (IR). The IR can code for an amino acid linker sequence that is used for direct connection of two GOI's (LINK11, LINK12).
Alternatively, the IR can code for a Shine Dalgarno (SD; prokaryotes) or IRES (eukaryotes) sequence for the construction of synthetic operons or promoters to ensure independent expression of the GOIs from one vector.

StarGate® fusion is realized by two sequential StarGate® transfer reactions:

In the first reaction, the two GOIs need to be transferred into special fusion vectors by two separate transfer reactions performed in parallel. The GOI that is intended to be positioned upstream in the final construct is transferred into a pNFUSE-IBA derivative, specifying the IR, while the second GOI is transferred into pCFUSE-IBA11 which is always the same irrespective of the desired IR.

In the second reaction, GOI1 and GOI2 cloned in the Fusion Vectors are assembled in a directed manner into pENTRY-IBA51 by a second one-tube reaction. An overview of fusion cloning is given by Figure 4A and a more detailed description is found in Figure 4B.

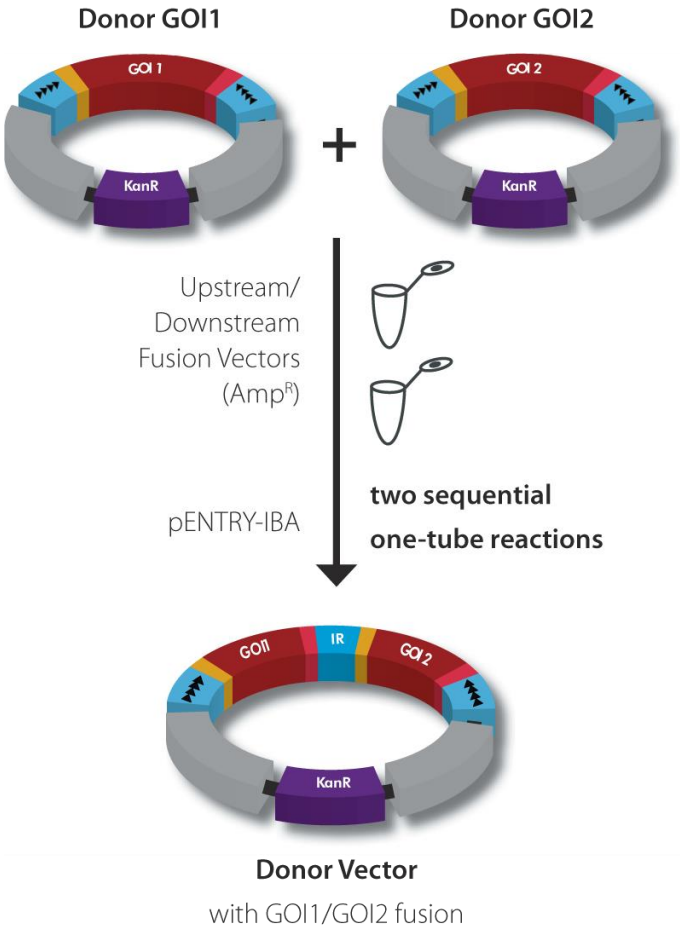


Fig. 4A
Two GOI's, GOI1 and GOI2, are fused by an intergenic region denoted by "IR".

For this purpose, one GOI, e.g., GOI1, is transferred into a pNFUSE-IBA derivative specifying upstream positioning while the other GOI, e.g., GOI2, is transferred in a separate reaction into pCFUSE-IBA11 specifying downstream positioning. In a subsequent step, GOI1 and GOI2 are assembled in pENTRY-IBA51 in a directed manner to connect them by an intergenic region IR as predetermined by the used pNFUSE-IBA derivative. A more detailed overview of the fusion reactions is given in Fig. 4B).

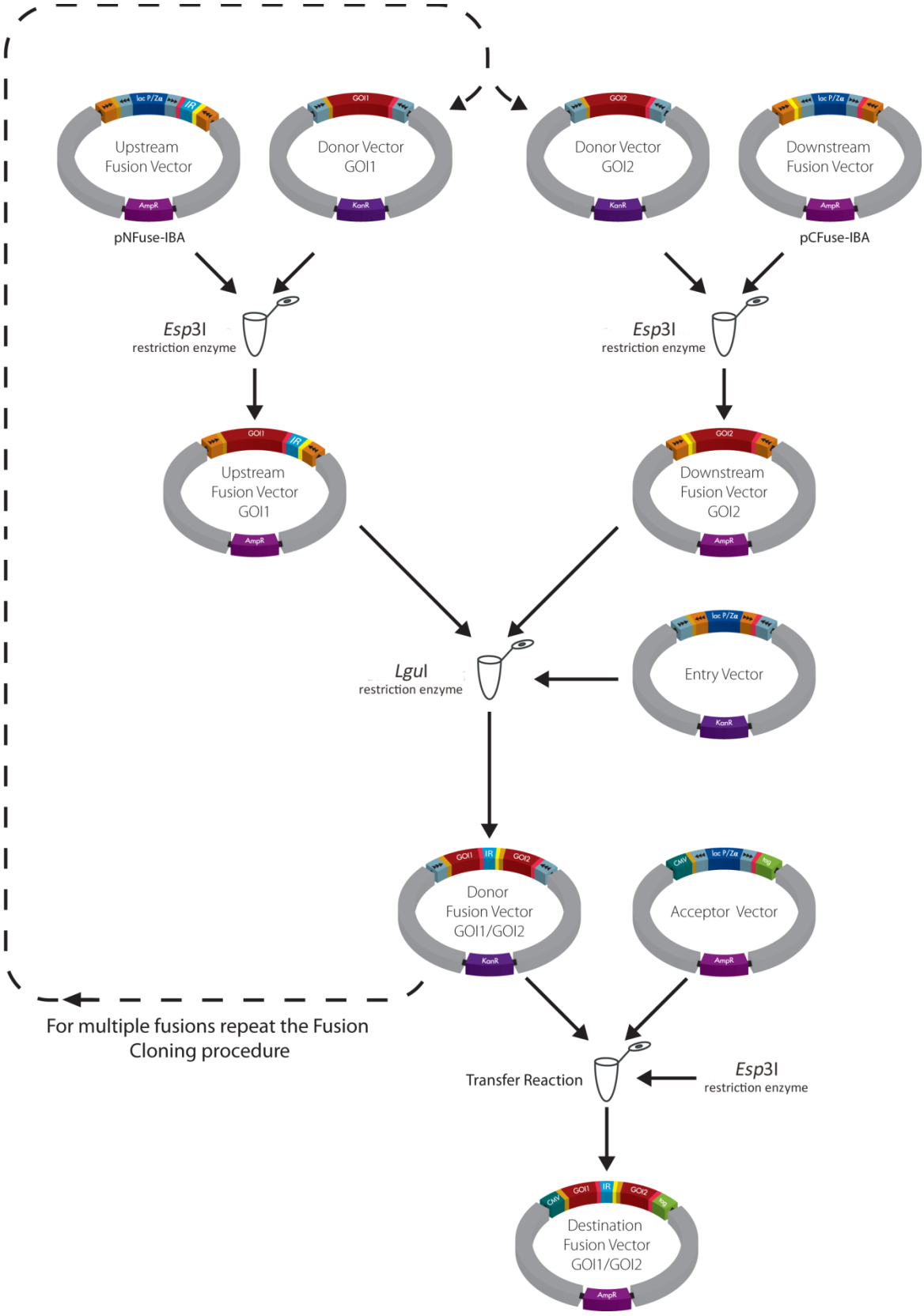


Fig. 4B
Detailed overview of StarGate® fusion cloning

2.4 Destination Vector Generation

The transfer of the GOI from the Donor Vector into a selected Acceptor Vector will lead to the generation of the Destination Vector, the final expression construct.
Acceptor Vectors provide the different genetic surroundings (i.e., purification tag, promoter, signal sequence, etc.; see 29). By mixing the Donor Vector with the respective Acceptor Vector the Destination Vector, final expression vector, is formed in a further one-tube reaction (Figure 5).

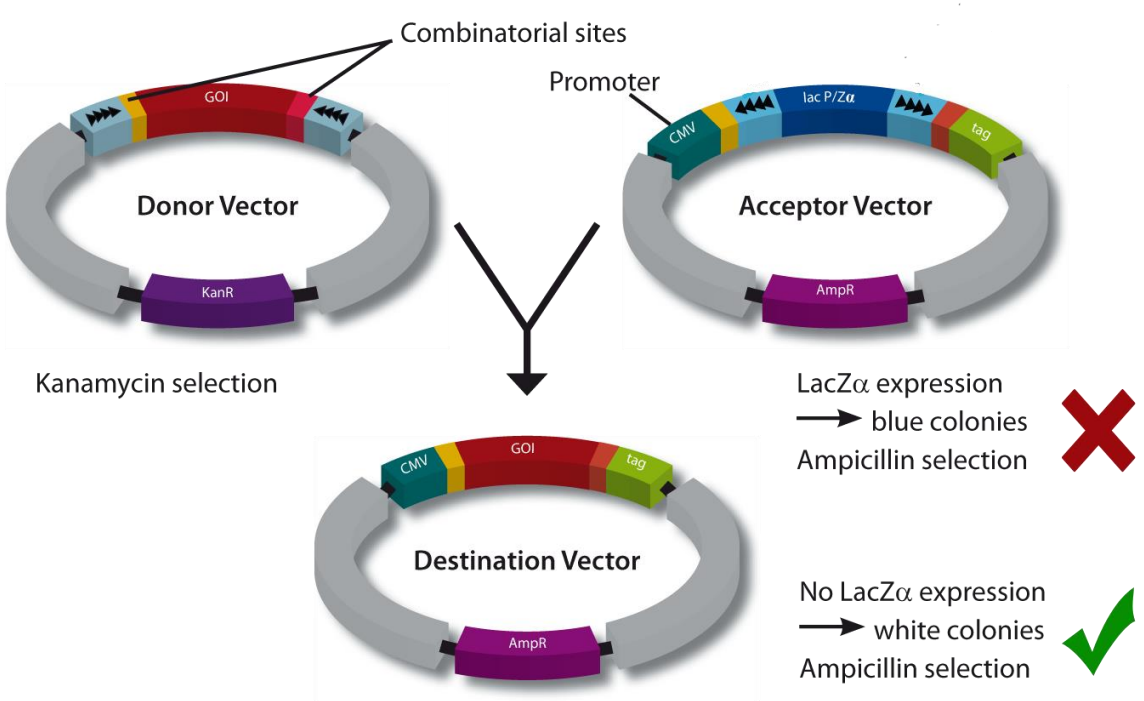


Fig. 5
Recombination will take place at the AATG and GGGA combinatorial sites, thereby imposing the occurrence of these sites in the final Destination Vector. The more complex recognition sites are eliminated and not expressed. Loss of the recognition sites drives the reaction towards generation of the desired Destination Vector.

E. coli is transformed with the mixture and plated on LB agar plates containing ampicillin and X-gal. Desired Destination Vectors including GOI will generate white colonies while undesirable Acceptor Vectors without GOI will generate blue colonies (Figure 6).

IBA also provides the possibility to directly clone a GOI into the Acceptor Vector. For this, use our manual “StarGate® – Direct Transfer Cloning”. For download go to: www.iba-lifesciences.com/technical-support.html.

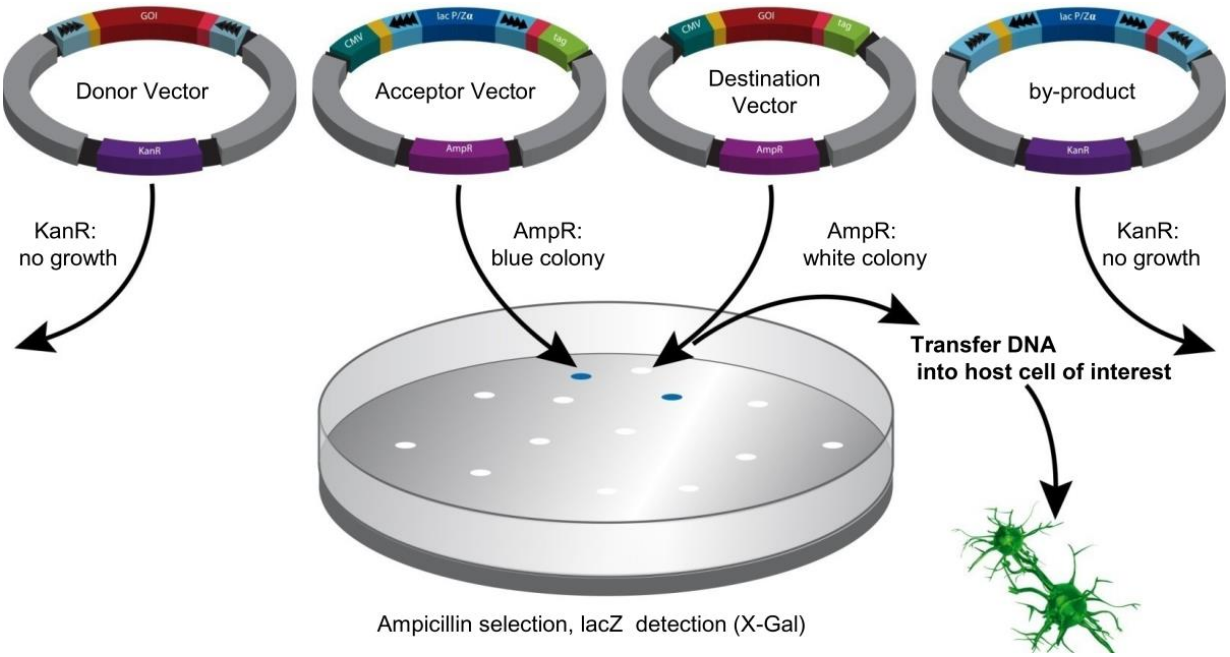


Fig. 6
E. coli is transformed with the mixture that potentially includes all 4 possible vector events. Due to selection on ampicillin plates, Donor Vector and by-product – which provide a kanamycin resistance only – will not enable growth of *E. coli*. Acceptor Vector and Destination Vector, however, enable growth due to the encoded ampicillin resistance genes. The Acceptor Vector without GOI carries the LacZα gene and, therefore, produces blue colonies on X-gal containing plates. LacZα has been replaced by GOI in the Destination Vector which, therefore, generates white colonies.

3 List of necessary components

3.1 Donor Vector generation

StarGate® Entry Cloning

IBA products	Cat.No.	
pENTRY-IBA51 The vector is provided as 5 µg aliquot [250 ng/µl].	5-4091-001	
Forward sequencing primer for pENTRY-IBA51 (HPLC purified)	5-0000-153	Combi-Pack: 5-0000-154
Reverse sequencing primer for pENTRY-IBA51 (HPLC purified)	5-0000-152	
Competent cells *E. coli* Top10 (100 µl)	5-1600-020	

Products from other suppliers
Pfu DNA Polymerase and corresponding PCR reagents
Primer set for amplification of the gene of interest and attachment of combinatorial sites
*Lgu*I [5 U/µl] restriction enzyme and supplied buffer (e.g. Thermo Scientific; The enzyme must be active at 37°C.)
ATP
T4 DNA Ligase [1 U/µl]
DNA-Ruler to determine the concentration of purified PCR-fragments

Additional materials required	• Thermocycler • Incubator • Agarose gel electrophoresis equipment • LB agar plates with 50 mg/l kanamycin and 50 mg/l X-gal • *Xba*I/*Hin*dIII restriction endonucleases

3.2 Fusion Cloning

IBA products	Cat.No.	
pENTRY-IBA51	5-4091-001	
pCFUSE-IBA11	5-1630-001	
Available pNFUSE vectors		
pNFUSE-IBA-IRES11	5-1631-001	
pNFUSE-IBA-SD11	5-1632-001	
pNFUSE-IBA-LINK11	5-1633-001	
pNFUSE-IBA-LINK12	5-1634-001	
The vectors are provided as 5 µg aliquots [250 ng/µl].		
Sequencing primers		
Forward sequencing primer for pCFUSE and pNFUSE (HPLC purified)	5-0000-155	Combi-Pack: 5-0000-157
Reverse sequencing primer for pCFUSE and pNFUSE (HPLC purified)	5-0000-156	
Forward sequencing primer for pENTRY-IBA51 (HPLC purified)	5-0000-153	Combi-Pack: 5-0000-154
Reverse sequencing primer for pENTRY-IBA51 (HPLC purified)	5-0000-152	
Competent cells *E. coli* Top10 (100 µl)	5-1600-020	

Products from other suppliers
Pfu DNA Polymerase and corresponding PCR reagents
Primer set for amplification of the gene of interest and attachment of combinatorial sites
*Lgu*I [5 U/µl] restriction enzyme and supplied buffer (e.g. Thermo Scientific; The enzyme must be active at 37°C.)
*Esp*3I [10 U/µl] restriction enzyme and supplied buffer (e.g. Thermo Scientific; The enzyme must be active at 37°C. Do not use the isoschizomer *Bsm*BI.)
ATP
DTT
T4 DNA Ligase [1 U/µl]

Additional materials required	• LB agar plates with 100 mg/L ampicillin and 50 mg/L X-gal • LB agar plates with 50 mg/L kanamycin and 50 mg/L X-gal

3.3 Destination Vector generation

StarGate® Transfer Cloning

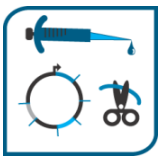
Acceptor Vector (Expression vector)
Acceptor Vectors with respective Cat. No. are listed on page 29 *ff*. The vectors are available as 5 µg aliquots [250 ng/µl].

Products from other suppliers
ATP
DTT
T4 DNA Ligase [1 U/µl]
*Esp*3I [10 U/µl] restriction enzyme and supplied buffer (e.g. Thermo Scientific; The enzyme must be active at 37°C. Do not use the isoschizomer *Bsm*BI.)

Complementary products	Cat.No.	
Competent cells *E. coli* TOP10 (20 rxns)	5-1600-020	
Sequencing primers for *E. coli* vectors		
Forward sequencing primer for pASG-IBA and pASK-IBA vectors (HPLC purified)	5-0000-101	Combi-Pack: 5-0000-104
Reverse sequencing primer for pASG-IBA and pASK-IBA vectors (HPLC purified)	5-0000-102	
Forward sequencing primer for pPSG-IBA and pPR-IBA vectors (HPLC purified)	5-0000-111	Combi-Pack: 5-0000-114
Reverse sequencing primer for pPSG-IBA and pPR-IBA vectors (HPLC purified)	5-0000-112	
Sequencing primers for mammalian vectors		
Forward sequencing primer for pESG-IBA, pCSG-IBA, pDSG-IBA and pEXPR-IBA vectors (HPLC purified)	5-0000-121	Combi-Pack: 5-0000-124
Reverse sequencing primer for pESG-IBA, pCSG-IBA, pDSG-IBA and pEXPR-IBA vectors (HPLC purified)	5-0000-122	
Sequencing primers for yeast vectors		
Forward sequencing primer for pYSG-IBA vector (HPLC purified)	5-0000-141	Combi-Pack: 5-0000-144
Reverse sequencing primer for pYSG-IBA vector (HPLC purified)	5-0000-142	
Sequencing primers for insect cells vectors		
Forward sequencing primer for pLSG-IBA vector (HPLC purified)	5-0000-161	Combi-Pack: 5-0000-164
Reverse sequencing primer for pLSG-IBA vector (HPLC purified)	5-0000-162	

Additional materials required	• Incubator • LB agar plates with 100 mg/l ampicillin and 50 mg/l X-gal

4 StarGate® protocols/recommendations



4.1 Donor Vector generation via Entry Cloning

4.1.1 PCR to amplify and equip GOI for subsequent cloning

4.1.1.1 Primer design

Important notes

- In case of using a proof reading 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 thereby impairing annealing efficiency and consequently the yield of the PCR product.
- Initial hybridizing regions of Primers (marked with | in the scheme below) should have a theoretical melting temperature between 60 °C and 63 °C. This will be achieved automatically if the StarPrimer D'Signer-Software is used. Otherwise 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).
- 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'-AGCGGCTCTTCAATGTTGACCTGCAACAGCTGCAT-3'
                  ||||||||||||||||||||
               5'-TTGACCTGCAACAGCTGCATAGCC-3'
               3'-AACTGGACGTTGTCGACGTATCGG-5'
                       ||||||||||||||||||||
                    3'-GACGTTGTCGACGTATCGGCCCTCTTCTCGGCGA-5'
```

(CR: reverse primer)

5' end of the CF primer (forward primer) is elongated by the AATG combinatorial site (italic and bold) and a sequence containing the *Lgu*I 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 a sequence containing the *Lgu*I recognition site (underlined).

Continue page 19

Important notes, continued

The resulting PCR product then has the following sequence:

```
5’-AGCGGCTCTTCAATGTTGACCTGCAACAGCTGCATAGCCGGGAGAAGAGCCGCT-3’
3’-TCGCCGAGAAGTTACAACTGGACGTTGTCGACGTATCGGCCCTCTTCTCGGCGA-5’
                 MetLeuThrCysAsnSerCysIleAlaGly
```

4.1.1.2 PCR amplification of the GOI

Important notes

Essential parameters for optimization are annealing temperature, duration of synthesis and template concentration.

Protocol

1. **Mix the following reagents in a 500 µl reaction tube and resulting in a total volume of 50 µl** (based on standard protocols for *Pfu* Polymerase PCR)**:**

200 µM	dNTP (each)
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) **0,1-1 ng/µl** (cDNA library)	Template DNA
2.5 U	*Pfu* DNA polymerase (depending on the recommendations of the manufacturer. *Pfu* can also be added after the initial denaturation step)
ad 50 µl	distilled H_2O

2. **Use a heated lid when available. Alternatively, overlay the sample with 50 µl mineral oil.**

3. **For initial denaturation heat the sample at 94 °C for 3 min.**

4. **Start temperature cycling:**

94 °C	30 s	Denaturation	Use 15 - 20 cycles for plasmid DNA and 30-40 cycles for cDNA library
55 - 65 °C	30 s - 1 min	Annealing	
72 °C	30 s - 4 min	DNA synthesis	

5. **Perform a final 60-72 °C incubation step for 5 min in order to obtain full length products.**

6. **Store samples at 4 °C until further analysis (e.g., agarose gel electrophoresis)**

4.1.1.3 Purification of PCR product

Protocol

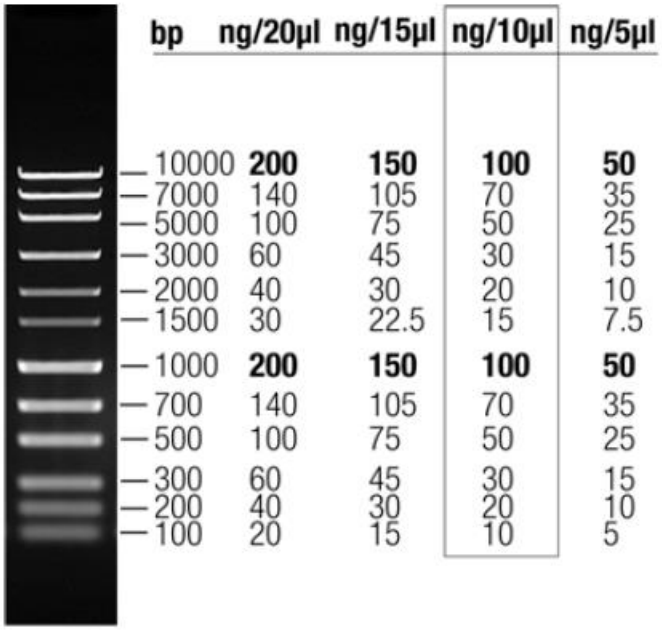
1. **Purify PCR product to remove *Pfu* polymerase and primers.**
   If PCR reaction produced a single product of the expected size the product can be purified using a clean up spin kit (according to the instructions of the manufacturer).
   If multiple bands are visible, it is recommended to isolate the PCR product by preparative gel electrophoresis.

2. **Quantify PCR fragment by analytical agarose gel electrophoresis through band intensity comparison with a DNA Ruler.**
   Applying two different amounts of PCR product in separate lanes is recommended to find a band of equal intensity with a band of a DNA Ruler which has to be applied on the same gel as internal standard for exact quantification.
   Determine PCR product concentration and dilute the PCR product to 2 nM with water (corresponds to 0.7 ng/µl for a 0.5 kb fragment, 1.4 ng/µl for a 1 kb fragment, 2.1 ng/µl for a 1.5 kb fragment, 2.8 ng/µl for a 2 kb fragment, 3.5 ng/µl for a 2.5 kb fragment, etc.).

3. **Alternatively: Determine the PCR product concentration using a NanoDrop.**

Example of a DNA Standard:

Apply 5 - 10 µl DNA Ruler per lane.



bp	ng/20µl	ng/15µl	ng/10µl	ng/5µl
10000	**200**	**150**	**100**	**50**
7000	140	105	70	35
5000	100	75	50	25
3000	60	45	30	15
2000	40	30	20	10
1500	30	22.5	15	7.5
1000	**200**	**150**	**100**	**50**
700	140	105	70	35
500	100	75	50	25
300	60	45	30	15
200	40	30	20	10
100	20	15	10	5

Fig. 7
DNA Ruler is a molecular size standard where each band represents a defined amount of linear DNA.

4.1.2 Reaction for Donor Vector generation

Protocol

1. **Dilute the provided pENTRY-IBA51 vector.** Pipette 2 µl vector [250 ng/µl] into 748 µl sterile water or buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) to obtain the required vector concentration [5 ng in 7.5 µl] for step 2.

2. **Mix the following reagents into a new reaction tube for insertion of the GOI into the Entry Vector** (In order to avoid evaporation and condensation in small reaction volumes, we recommend to use 200 µl reaction tubes like e.g. Sarstedt Multiply, or incubation in 30 °C incubators instead of thermo block or water bath)**:**

7.5 µl	Entry Vector pENTRY-IBA51 [5 ng]
12 µl	PCR product (2 nM) from 4.1.1.2
1 µl	12.5 mM ATP
1 µl	T4-DNA-Ligase [1 U/µl]
1 µl	*Lgu*I restriction enzyme [5 U/µl]
2.5 µl	Buffer supplied with *Lgu*I

3. **Close the reaction vessel thoroughly, mix gently and incubate for 1 h at 30 °C.**

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

5. **After incubation, add a 10 µl aliquot of from the reaction mixture (25 µl) from step 2 to the thawed competent *E. coli* cells.**

6. **Mix gently (do not vortex) and incubate subsequently for 30 min on ice.**

7. **Mix gently (do not vortex) and incubate subsequently for 5 min at 37 °C.**

8. **Mix gently (do not vortex) and incubate subsequently 2-5 min on ice.**

9. **Add 900 µl LB medium and shake for 45 min at 37 °C.**

   Caution: This incubation step is necessary to express kanamycin resistance prior to plating on kanamycin plates for selection.

10. **Plate 100 µl on LB agar containing 50 mg/l kanamycin and 50 mg/l X-gal.**

11. **Centrifuge the residual 900 µl cell mixture for 30 sec in a microfuge, resuspend the cell sediment with 100 µl LB medium and plate the whole amount on a separate plate LB/kan/X-gal** (see step 10)**.**

12. **Incubate plates over night at 37 °C.**

4.1.3 Donor Vector identification

Protocol

1. **Pick 5 white colonies, cultivate in LB containing 50 mg/l kanamycin and perform DNA mini preparation.**

2. **Perform analytical *Xba*I/*Hin*dIII restriction.**
   A fragment with the length of the PCR product from 4.1.1.1 plus 40 bases is expected. (Check your GOI for internal *Xba*I/*Hin*dIII restriction sites and consider the changed DNA fragment sizes resulting from additional restriction sites).

3. **Select a putatively correct clone and confirm sequence via Donor Vector forward and reverse sequencing.**
   Sequencing is recommended as PCR may lead to mutations and to improper product ends. Appropriate primers are available (ENTRY-Primer-for2 and ENTRY-Primer-rev; listed above).

   The region flanking the GOI should have the sequence:

   TCTAGAAAAGCGCGTCTCC***AATG***–GOI–***GGGA***GGAGACGCGCTAAAAGCTT
   XbaI HindIII

4. **Dilute 1 µg of the verified Donor Vector plasmid DNA with water to a final concentration of 2 ng/µl and store at – 20 °C.**

4.2 Fusion Cloning procedure

4.2.1 Transfer of GOI's into Fusion Vectors

Important notes

The GOI, which will be located upstream in the final fusion construct, has to be transferred into the pNFUSE-IBA derivative, carrying the desired intergenic region (IR; see page 41). The GOI, which will be located downstream in the final construct has to be transferred into pCFUSE-IBA11. Perform the following transfer reaction for both GOI's in parallel with the respective Fusion vectors.

Protocol

1. **Dilute the provided vectors.** Pipette 2 µl vector [250 ng/µl] into 798 µl sterile water or buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) to obtain the required vector concentration [5 ng in 8 µl] for step 2.

2. **Mix the reagents below to the supplied reaction tube containing 10 µl of the appropriate Fusion Vector** (In order to avoid evaporation and condensation in small reaction volumes, we recommend to use 200 µl reaction tubes like e.g. Sarstedt Multiply, or incubation in 30 °C incubators instead of thermo block or water bath)**:**

8 µl	pNFUSE-IBA or pCFUSE-IBA, respectively [5 ng/µl]
12 µl	Diluted Donor Vector solution (2 ng/µl; see 4.1.3 or 4.2.3)
1 µl	DTT/ATP mix [250 mM DTT; 12.5 mM ATP]
1 µl	T4-DNA-Ligase [1 U/µl]
0.5 µl	*Esp*3I [10 U/µl]
2.5 µl	Buffer supplied with *Esp*3I

3. **Close the reaction vessel thoroughly, mix gently and incubate at 30 °C for 1 h.**

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

5. **After incubation, add an aliquot of 10 µl from the reaction mixture (25 µl) from step 2 to the thawed competent *E. coli* cells.**
   Continue incubation of the residual reaction mixture (15 µl) in the refrigerator at 2-8 °C for backup purposes.

6. **Mix gently (do not vortex) and incubate subsequently for 30 min on ice.**

7. **Mix gently (do not vortex) and incubate subsequently for 5 min at 37 °C.**

8. **Mix gently (do not vortex) and incubate subsequently 2-5 min on ice.**

Continue on page 24

Protocol, continued

9. **Plate 10 µl (mixed with 90 µl LB medium) and 100 µl on two separate LB agar plates containing 100 mg/L ampicillin and 50 mg/L X-gal.**

10. **Incubate plates over night at 37 °C.**

4.2.2 Recombinant Fusion Vector identification

Protocol

1. **Pick 3 white colonies and perform DNA mini preparation.**

2. **Upstream and downstream Fusion vectors have *Xba*I/*Hin*dIII restriction sites that flank the GOI insertion site and can be used for confirmation of GOI integration:**

   a. In case of the downstream Fusion vector pCFUSE-IBA11, a DNA fragment with the length of GOI (without start and stop codon) plus 36 bases will be generated.

   b. In case of the upstream Fusion vectors, a DNA fragment with the length of GOI (without start and stop codon) plus the length of the respective intergenic region (IR; please refer to the respective vector data sheet) plus 34 bases will be generated.

   Check your GOI for internal *Xba*I/*Hin*dIII restriction sites and consider the changed DNA fragment sizes resulting from additional sites.

3. **Dilute 1 µg of the verified Donor Vector plasmid DNA with water to a final concentration of 2 ng/µl and store at – 20 °C.**

4.2.3 Assembly of GOI1 and GOI2 in pENTRY-IBA51

Protocol

1. **Mix the following reagents into a new reaction tube** (In order to avoid evaporation and condensation in small reaction volumes, we recommend to use 200 µl reaction tubes like e.g. Sarstedt Multiply, or incubation in 30 °C incubators instead of thermo block or water bath)**:**

8 µl	Entry Vector pENTRY-IBA51 [5 ng]
6 µl	Diluted upstream Fusion Vector pNFUSE-IBA with GOI1 (4 ng/µl) from 4.2.2
6 µl	Diluted downstream Fusion Vector pCFUSE-IBA with GOI2 (4 ng/µl) from 4.2.2
1 µl	ATP [12.5 mM ATP]
1 µl	T4-DNA-Ligase [1 U/µl]
0.5 µl	*Lgu*I [5 U/µl]
2.5 µl	Buffer supplied with *Lgu*I

Continue on page 25

Protocol (continued)

2. **Close the reaction vessel thoroughly, mix gently and incubate for 1 h at 30 °C.**

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

4. **After incubation, add an aliquot of 10 µl from the reaction mixture (25 µl) from step 2 to the thawed competent *E. coli* cells.**
   Continue incubation of the residual reaction mixture (15 µl) in the refrigerator at 2-8 °C for backup purposes.

5. **Mix gently (do not vortex) and incubate subsequently for 30 min on ice.**

6. **Mix gently (do not vortex) and incubate subsequently for 5 min at 37 °C.**

7. **Mix gently (do not vortex) and incubate subsequently 2-5 min on ice.**

8. **Add 900 µl LB medium and shake for 45 min at 37 °C.**

   
   Caution: This incubation step is necessary to express kanamycin resistance prior to plating on kanamycin plates for selection.

9. **Plate 100 µl on LB agar containing 50 mg/l kanamycin and 50 mg/l X-gal.**

10. **Centrifuge the residual 900 µl cell mixture for 30 sec in a microfuge, resuspend the cell sediment with 100 µl LB medium and plate the whole amount on a separate plate LB/kan/X-gal** (see step 9)**.**

11. **Incubate plates over night at 37 °C.**

4.2.4 Donor Vector identification (fused GOI1 + GOI2)

Protocol

1. **Pick 5 white colonies and perform DNA mini preparation.**

2. **Perform *Xba*I/*Hin*dIII restriction digestion.**
   A fragment with the length of mutated GOI plus 40 bases is expected (Check your GOI for internal *Xba*I/*Hin*dIII restriction sites and consider the changed DNA fragment sizes resulting from additional sites).

3. **Select a putatively correct clone and optionally confirm the sequence using Donor Vector forward and reverse sequencing primers (ENTRY-Primer-for2 and ENTRY-Primer-rev).**

Continue on page 26

Protocol, continued

The combinatorial site flanking region should have the sequence:

TCTAGAAAAGCGCGTCTCC***AATG***-GOI1-***GGGA***-IR-***AATG***-GOI2-***GGGA***GGAGACGCGCTAAAAGCTT
*Xba*I *Hin*dIII

Possible intergenic region (IR) sequences can be found on our homepage.

4. **Dilute 1 µg of the verified Donor Vector plasmid DNA with water to a final concentration of 2 ng/µl and store at -20 °C.**

4.3 Destination Vector generation

4.3.1 GOI transfer reaction

Important notes	If a GOI should be directly transferred into the Acceptor Vector, use our manual "StarGate – Direct Transfer Cloning". For download go to: www.iba-lifesciences.com/technical-support.html. This direct method is recommended, if something is known about the required expression conditions of the respective GOI.

Protocol

1. **Dilute the provided Acceptor Vector.** Pipette 2 µl vector [250 ng/µl] into 748 µl sterile water or buffer (10 mM Tris/HCl pH 8.0, 1 mM EDTA) to obtain the required vector concentration [5 ng in 7.5 µl] for step 2.

2. **Mix the reagents below into a new reaction tube:**

7.5 µl	Acceptor Vector of choice [5 ng; dilute in distilled water for correct concentration]
12.5 µl	Diluted Donor Vector solution containing GOI (2 ng/µl)
1 µl	DTT/ATP mix [250 mM DTT; 12.5 mM ATP]
1 µl	T4-DNA-Ligase [1 U/µl]
0.5 µl	*Esp*3I [10 U/µl]
2.5 µl	Buffer supplied with *Esp*3I

3. **Close the reaction vessel thoroughly, mix gently and incubate at 30 °C for 1 h.**

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

5. **After incubation, take a 10 µl aliquot from the reaction mixture (25 µl) from step 2 and add it to the thawed competent *E. coli* cells.**
   Continue incubation of the residual reaction mixture (15 µl) in the refrigerator at 2-8 °C for backup purposes.

6. **Mix gently (do not vortex) and incubate subsequently for 30 min on ice.**

7. **Mix gently (do not vortex) and incubate subsequently for 5 min at 37 °C.**

8. **Mix gently (do not vortex) and incubate subsequently 2-5 min on ice.**

9. **Plate 10 µl (mixed with 90 µl LB medium) and 100 µl on LB agar containing 100 mg/l ampicillin and 50 mg/l X-gal.**

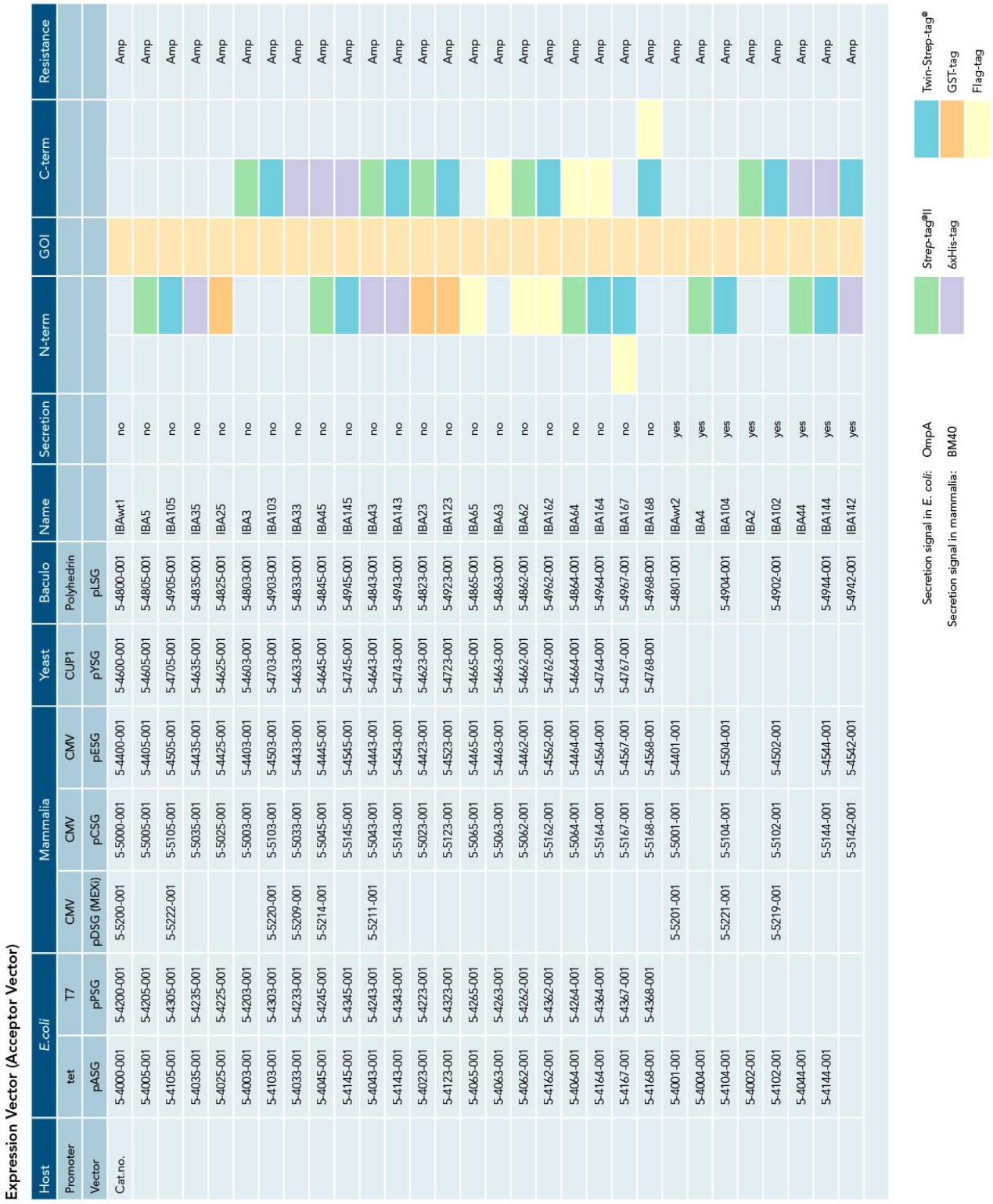
10. **Incubate plates over night at 37 °C.**

4.3.2 Destination Vector identification

Protocol	**1.** Pick 3 white colonies and perform DNA mini preparation. **2.** pASG-IBA, pPSG-IBA, pDSG-IBA, pESG-IBA, pCSG-IBA and pYSG-IBA have *Xba*I/*Hin*dIII restriction sites that flank the expression cassette and, therefore, may be used for confirmation of GOI integration. **3.** Due to an additional *Hin*dIII site downstream to GOI, an additional fragment of 456 bp will be generated after *Xba*I/*Hin*dIII cleavage of pLSG-IBA vectors. **4.** For exact calculation of expected restriction fragment length please refer to the appropriate Acceptor Vector data sheet.

5 StarGate® Acceptor Vector collection description

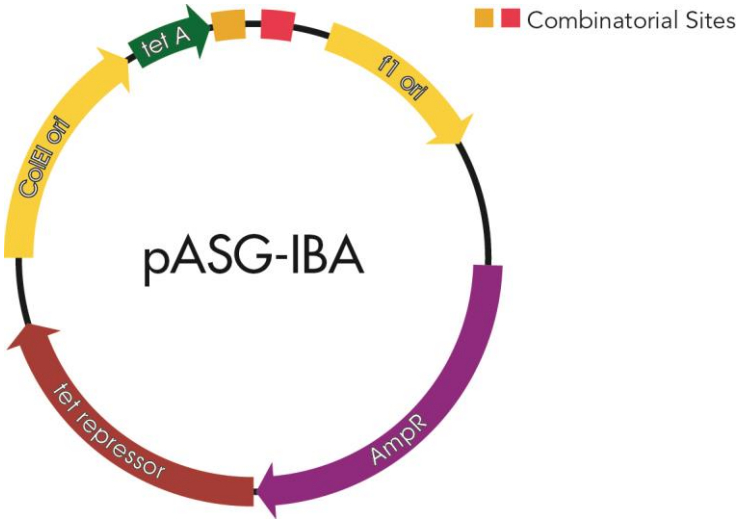
5.1 Overview



Cat.no.	pASG	pPSG	pDSG (MEXi)	pCSG	pESG	pYSG	pLSG	Name	Secretion	N-term	GOI	C-term	Resistance
	5-4000-001	5-4200-001	5-5200-001	5-5000-001	5-4400-001	5-4600-001	5-4800-001	IBAwt1	no				Amp
	5-4005-001	5-4205-001		5-5005-001	5-4405-001	5-4605-001	5-4805-001	IBA5	no				Amp
	5-4105-001	5-4305-001	5-5222-001	5-5105-001	5-4505-001	5-4705-001	5-4905-001	IBA105	no				Amp
	5-4035-001	5-4235-001		5-5035-001	5-4435-001	5-4635-001	5-4835-001	IBA35	no				Amp
	5-4025-001	5-4225-001		5-5025-001	5-4425-001	5-4625-001	5-4825-001	IBA25	no				Amp
	5-4003-001	5-4203-001		5-5003-001	5-4403-001	5-4603-001	5-4803-001	IBA3	no				Amp
	5-4103-001	5-4303-001	5-5220-001	5-5103-001	5-4503-001	5-4703-001	5-4903-001	IBA103	no				Amp
	5-4033-001	5-4233-001	5-5209-001	5-5033-001	5-4433-001	5-4633-001	5-4833-001	IBA33	no				Amp
	5-4045-001	5-4245-001	5-5214-001	5-5045-001	5-4445-001	5-4645-001	5-4845-001	IBA45	no				Amp
	5-4145-001	5-4345-001		5-5145-001	5-4545-001	5-4745-001	5-4945-001	IBA145	no				Amp
	5-4043-001	5-4243-001	5-5211-001	5-5043-001	5-4443-001	5-4643-001	5-4843-001	IBA43	no				Amp
	5-4143-001	5-4343-001		5-5143-001	5-4543-001	5-4743-001	5-4943-001	IBA143	no				Amp
	5-4023-001	5-4223-001		5-5023-001	5-4423-001	5-4623-001	5-4823-001	IBA23	no				Amp
	5-4123-001	5-4323-001		5-5123-001	5-4523-001	5-4723-001	5-4923-001	IBA123	no				Amp
	5-4065-001	5-4265-001		5-5065-001	5-4465-001	5-4665-001	5-4865-001	IBA65	no				Amp
	5-4063-001	5-4263-001		5-5063-001	5-4463-001	5-4663-001	5-4863-001	IBA63	no				Amp
	5-4062-001	5-4262-001		5-5062-001	5-4462-001	5-4662-001	5-4862-001	IBA62	no				Amp
	5-4162-001	5-4362-001		5-5162-001	5-4562-001	5-4762-001	5-4962-001	IBA162	no				Amp
	5-4064-001	5-4264-001		5-5064-001	5-4464-001	5-4664-001	5-4864-001	IBA64	no				Amp
	5-4164-001	5-4364-001		5-5164-001	5-4564-001	5-4764-001	5-4964-001	IBA164	no				Amp
	5-4167-001	5-4367-001		5-5167-001	5-4567-001	5-4767-001	5-4967-001	IBA167	no				Amp
	5-4168-001	5-4368-001		5-5168-001	5-4568-001	5-4768-001	5-4968-001	IBA168	no				Amp
	5-4001-001		5-5201-001	5-5001-001	5-4401-001		5-4801-001	IBAwt2	yes				Amp
	5-4004-001							IBA4	yes				Amp
	5-4104-001		5-5221-001	5-5104-001	5-4504-001		5-4904-001	IBA104	yes				Amp
	5-4002-001							IBA2	yes				Amp
	5-4102-001		5-5219-001	5-5102-001	5-4502-001		5-4902-001	IBA102	yes				Amp
	5-4044-001							IBA44	yes				Amp
	5-4144-001			5-5144-001	5-4544-001		5-4944-001	IBA144	yes				Amp
				5-5142-001	5-4542-001		5-4942-001	IBA142	yes				Amp

Table 1: **IBA Expression Vector (Acceptor Vector) Overview.** A detailed overview of the current Acceptor Vectors is given here. The 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) e.g. IBA5 = *Strep*-tag®II/N-term/no secretion signal).

5.2 pASG-IBA



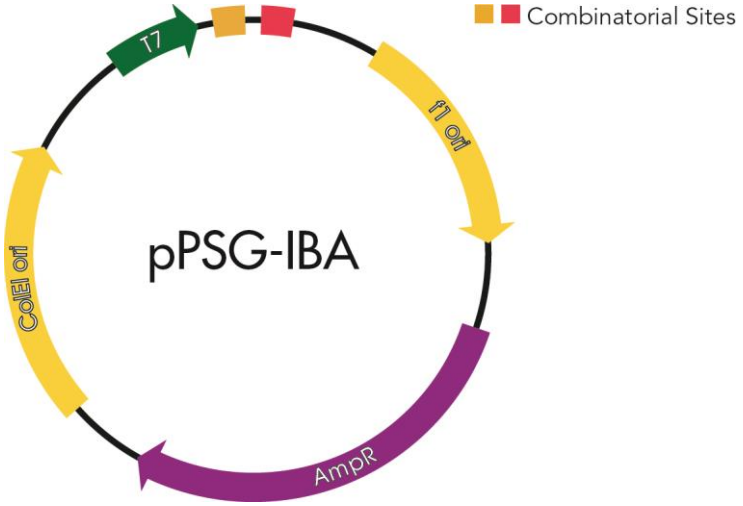
The production of a heterologous protein is often accompanied by an impaired growth of *E. coli* cells. Consequently, regulation of heterologous biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor. If the foreign protein is cytotoxic, even the production of minute quantities can result in a dramatic selection against the *E. coli* cells which harbor the expression plasmid. In such cases, tight repression of the promoter is required. Synthesis of the gene product is then switched on in a controlled manner simply by adding a chemical inducer. pASG-IBA vectors which are similar to pASK-IBA vectors carry the promoter/operator region from the *tet*A resistance gene and are the optimal solution for such an inducible expression system (Skerra, 1994). The strength of the *tet*A promoter is comparable with that of the *lac*-UV5 promoter. Some vectors carry the ompA signal sequence for secretion of the recombinant protein into the periplasmic space which is crucial for functional expression of proteins with structural disulfide bonds.

The *tet* promoter can be fully induced by adding anhydrotetracycline at a concentration that is not antibiotically effective (200 ng/ml). The constitutive expression of the *tet* repressor gene, which is also encoded on the expression plasmids, guarantees the repression of the promoter in the absence of the inducer. In a Western blot, no expression is detectable under these conditions (Skerra, 1994). In contrast to the *lac* promoter, which is susceptible to catabolite repression (cAMP-level, metabolic state) and chromosomally encoded repressor molecules, the *tet*A promoter/operator is not coupled to any cellular regulation mechanisms. Therefore, when using the *tet* system, there are basically no restrictions in the choice of culture medium or *E. coli* expression strain. For example, glucose minimal media and even the bacterial strain XL1-Blue, which carries an episomal copy of the tetracycline resistance gene, can be used for expression.

Plasmid propagation is supported by a ColEI ori (pUC) and the ampicillin resistance gene.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

5.3 pPSG-IBA



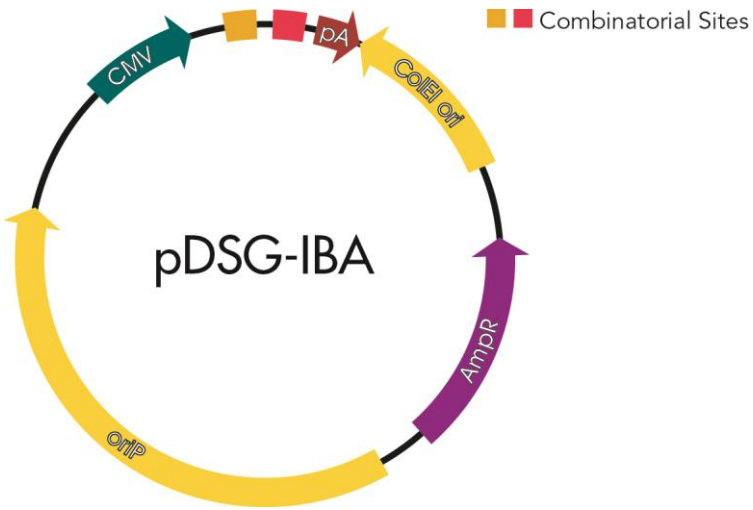
pPSG-IBA vectors which are similar to pPR-IBA vectors use the T7 promoter and T7 RNA polymerase for high-level transcription of the gene of interest (Studier *et al.*, 1990). As the T7 promoter is stronger than the *tet* promoter, pPSG-IBA vectors are recommended in cases where expression with the *tet* promoter does not lead to significant yields of the recombinant protein. In other cases, strong T7 expression may cause insoluble inclusion bodies. In such cases the *tet* promoter might be a good alternative when expression of soluble protein is desired.

Expression of the target genes is induced by providing a source of T7 RNA polymerase in the *E. coli* host cell. This is accomplished by using, e.g., an *E. coli* host which contains a chromosomal copy of the T7 RNA polymerase gene (e.g., BL21(DE3) which has the advantage to be deficient of *lon* and *ompT* proteases). The T7 RNA polymerase gene in BL21(DE3) is under control of the lacUV5 promoter which can be induced by addition of IPTG.

Plasmid propagation is supported by a ColEI ori (pUC) and the ampicillin resistance gene.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

5.4 pDSG-IBA



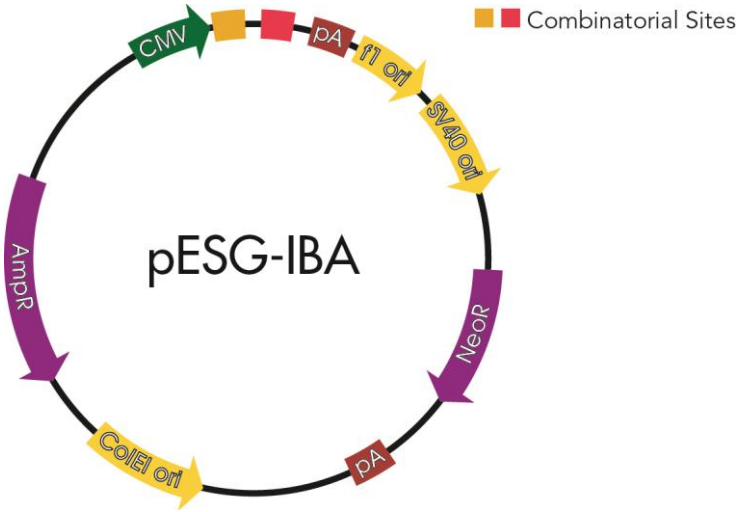
pDSG-IBA vectors are designed for high-level constitutive expression of recombinant proteins in a wide range of mammalian host cells through the human cytomegalovirus immediate-early (CMV) promoter (Boshart *et al*., 1985; Nelson *et al*., 1987). The Epstein Barr Virus replication origin (*oriP*) provides extrachromosomal replication in human, primate and canine cells which have the nuclear antigen encoded by EBNA-1 chromosomally expressed.

Propagation in *E. coli* is supported by a ColEI ori and the ampicillin resistance gene. Some vectors carry the BM40 signal sequence for secretion of the recombinant protein into the medium.

An advantage compared to pCSG is the small size of the vector.

Note: Please refer to the corresponding data sheet for detailed information on a given vector.

5.5 pESG-IBA

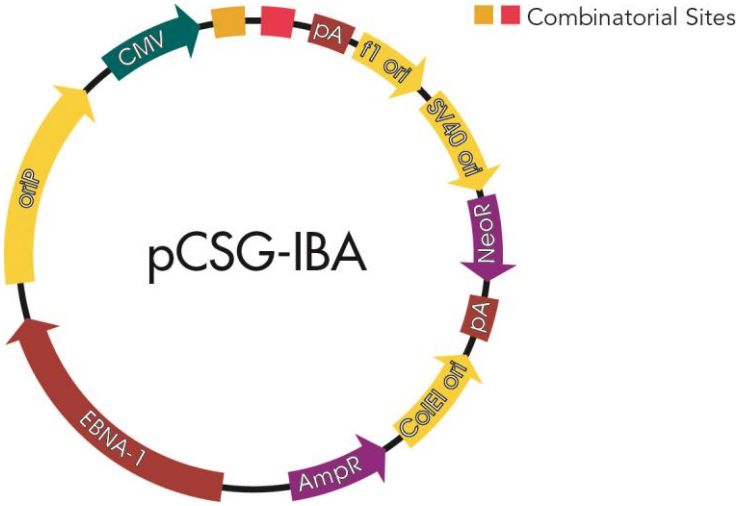


pESG-IBA vectors are designed for high-level constitutive expression of recombinant proteins in a wide range of mammalian host cells through the human cytomegalovirus immediate-early (CMV) promoter (Boshart *et al.*, 1985; Nelson *et al.*, 1987). To prolong expression in transfected cells, the vector will replicate in cell lines that are latently infected with SV40 large T antigen (e.g., COS1 or COS7). In addition, Neomycin resistance gene allows direct selection of stable cell lines.

Propagation in *E. coli* is supported by a ColEI ori and the ampicillin resistance gene. Some vectors carry the BM40 signal sequence for secretion of the recombinant protein into the medium.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

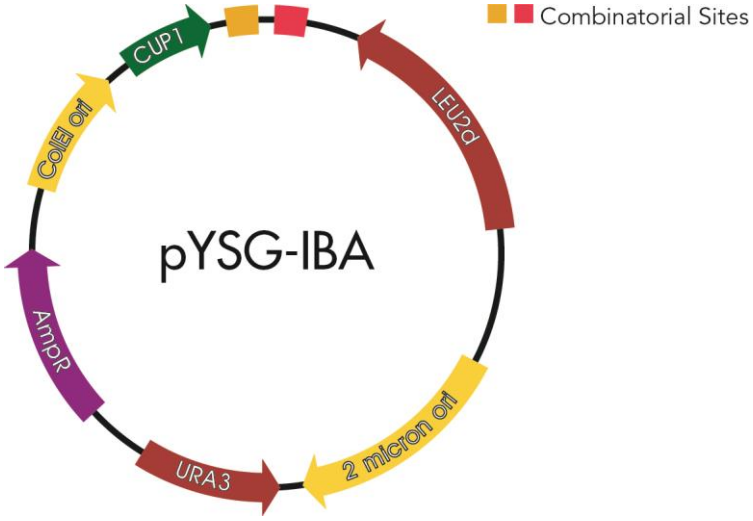
5.6 pCSG-IBA



pCSG-IBA vectors are designed for high-level constitutive expression of recombinant proteins in a wide range of mammalian host cells through the human cytomegalovirus immediate-early (CMV) promoter (Boshart *et al.*, 1985; Nelson *et al.*, 1987). The Epstein Barr Virus replication origin (oriP) and nuclear antigen encoded by EBNA-1 provide extrachromosomal replication in human, primate and canine cells and the SV40 replication origin provides extrachromosomal replication in cell lines that express SV40 large T antigen (e.g., COS1 or COS7). Thus, by means of the NeoR marker, prolonged expression of the inserted GOI occurs in such cell lines under G418 selection without the need for making stable cell lines. Propagation in *E. coli* is supported by a ColEI ori (pUC) and the ampicillin resistance gene. Finally, some vectors carry the BM40 signal sequence for secretion of the recombinant protein into the medium.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

5.7 pYSG-IBA



pYSG-IBA expression vectors are designed for high-level expression of recombinant proteins in yeast. Cloned genes are under the control of the Cu^{++}-inducible CUP1 promoter which means that expression is induced upon addition of copper sulfate. pYSG-IBA vectors favour correct protein folding and the production of soluble proteins — inclusion bodies rarely form.

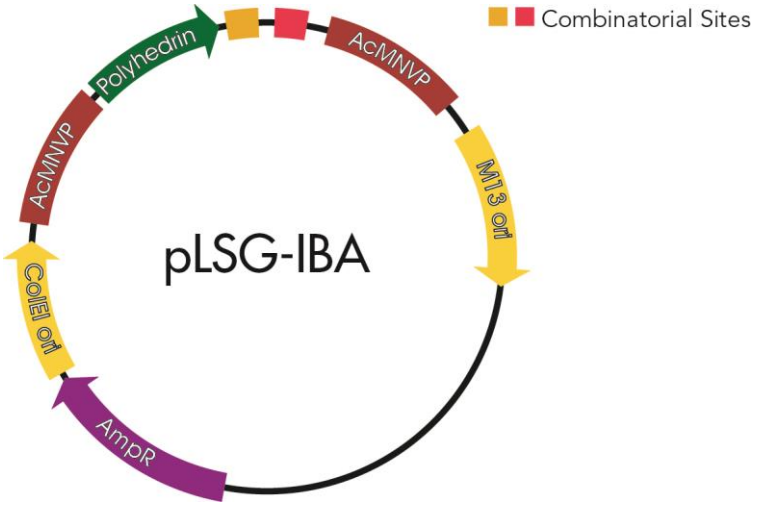
In addition, all vectors include the yeast selectable markers leu2-d (a LEU2 gene with a truncated, but functional promoter) and URA3. Vectors including the leu2-d marker are maintained at high copy number to provide enough gene products from the inefficient promoter for cell survival during growth selection in minimal medium lacking leucine (Macreadie *et al.*, 1991; Gietz & Sugino, 1989). For selection after transformation, the URA3 marker should be used instead of leu2-d to enable growth of transformants.

Optimal repression under non-inducing conditions is obtained with yeast strains carrying multiple $CUP1^r$ loci, while partially constitutive expression in strains lacking the $CUP1^r$ locus (ΔCUP1) is still enhanced upon the addition of copper through a trans-acting factor (Butt & Ecker, 1987).

Propagation in *E. coli* is supported by a ColEI ori (pUC) and the ampicillin resistance gene.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

5.8 pLSG-IBA



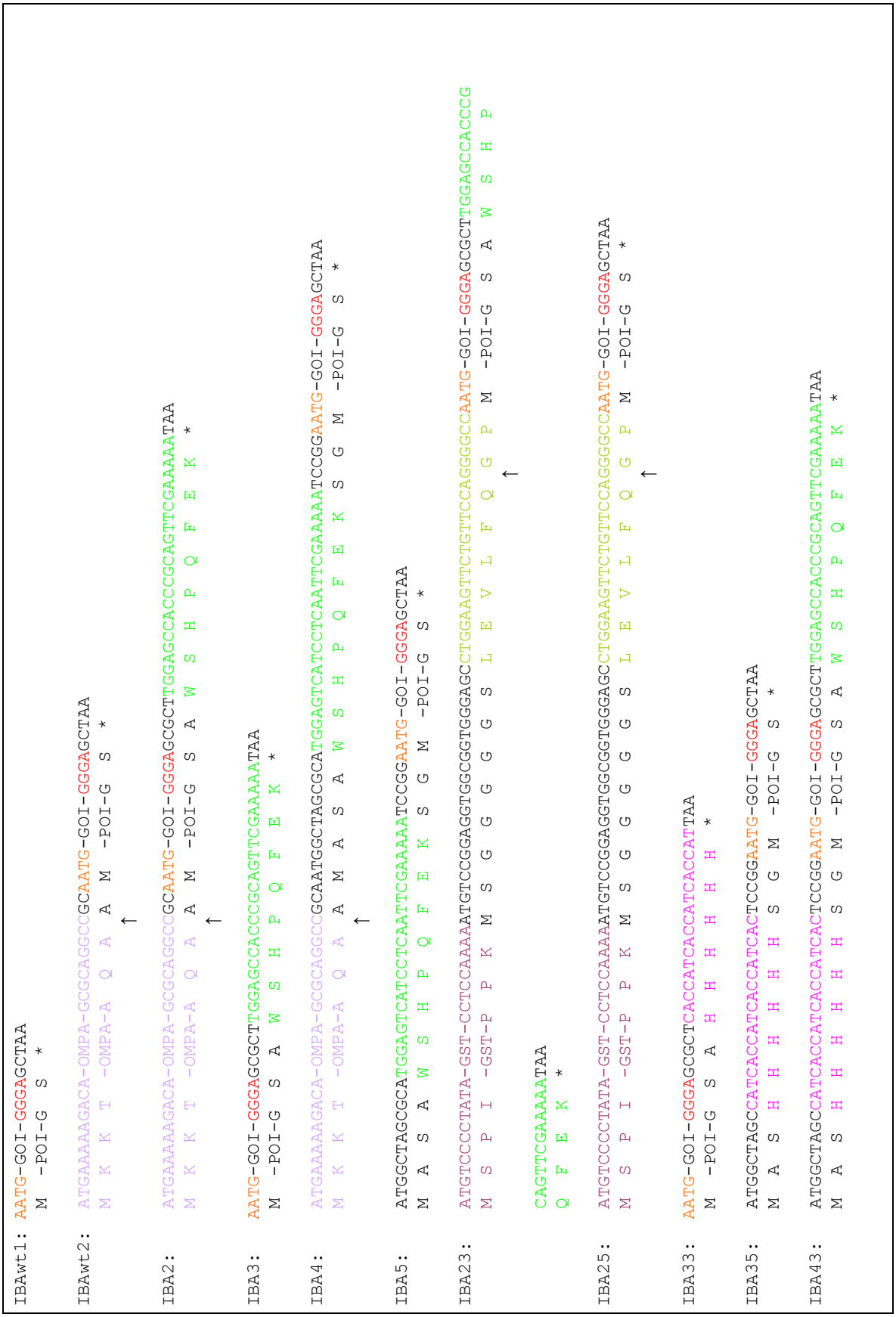
pLSG-IBA vectors are transfer vectors to introduce the GOI into the polyhedrin gene locus of AcMNPV DNA by homologous recombination. Co-transfection with BacPAK6 linearized AcMNPV DNA (Clontech) or circular *flash*BAC modified AcMNPV DNA (Oxford Expression Technologies) allows the generation of recombinant baculovirus at very high efficiency through reconstitution of an essential gene (ORF 1629) and elimination of wild type virus to great extent.

pLSG-IBA vectors provide the strong polyhedrin promoter for high level expression of an inserted GOI in insect cells.

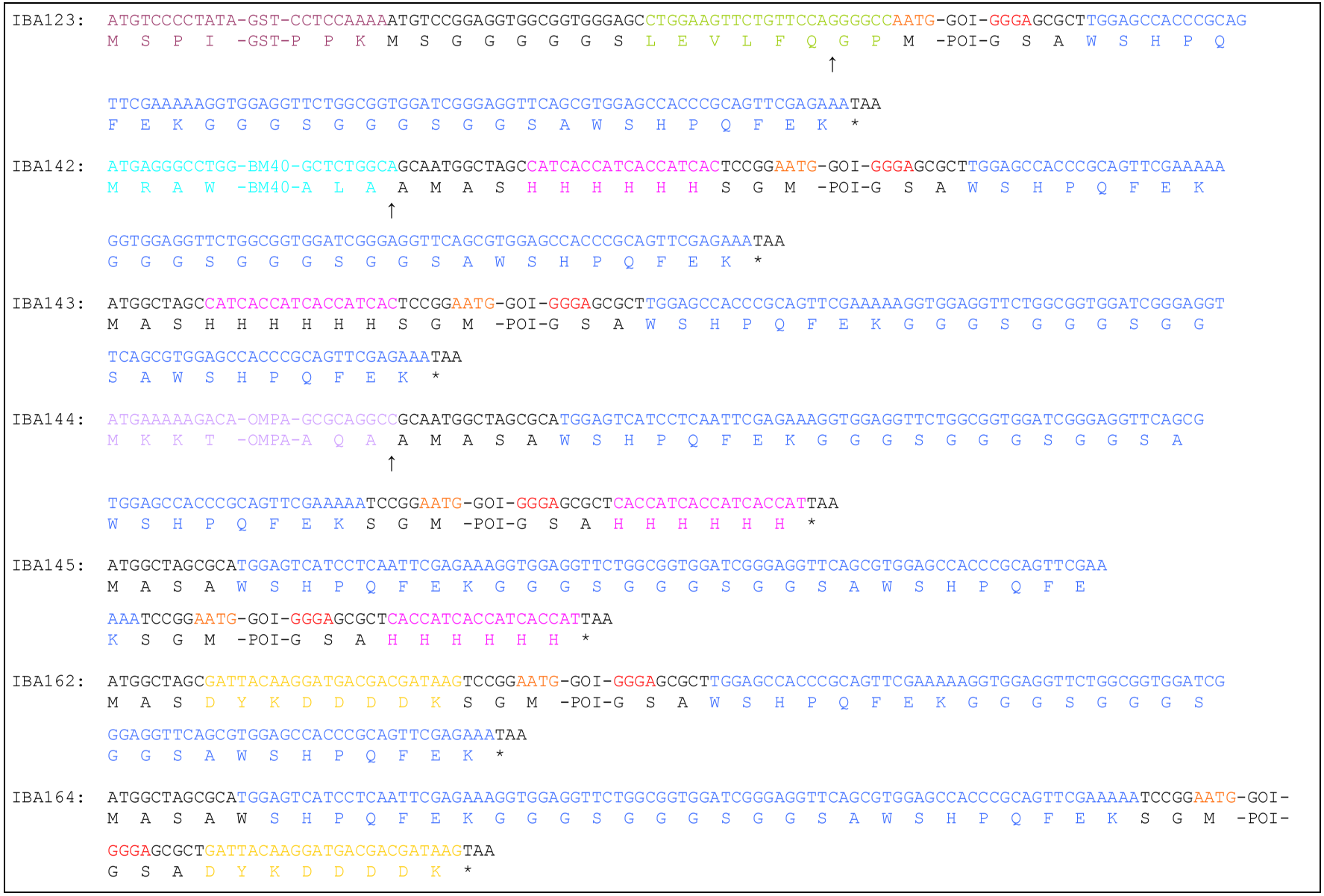
Propagation in *E. coli* is supported by a ColEI ori (pUC) and the ampicillin resistance gene.

Note: Please refer to the corresponding data sheet for detailed information on a given vector

6 StarGate® Acceptor Vector expression cassettes



IBA44:
ATGAAAAAGACA-OMPA-GCGCAGGCCGCAATGGCTAGCGCATGGAGTCATCCTCAATTCGAAAAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCAT
M K K T -OMPA-A Q A A M A S A W S H P Q F E K S G M -POI-G S A H H H H H
CACCATTAA
H H *
IBA45:
ATGGCTAGCGCATGGAGTCATCCTCAATTCGAAAAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
M A S A W S H P Q F E K S G M -POI-G S A H H H H H H *
IBA62:
ATGGCTAGCGATTACAAGGATGACGACGATAAGTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAATAA
M..A S D Y K D D D D K S G M -POI-G S A W S H P Q F E K *
IBA63:
AATG-GOI-GGGAGCGCTGATTACAAGGATGACGACGATAAGTAA
M -POI-G S A D Y K D D D D K *
IBA64:
ATGGCTAGCGCATGGAGTCATCCTCAATTCGAAAAATCCGGAATG-GOI-GGGAGCGCTGATTACAAGGATGACGACGATAAGTAA
M A S A W S H P Q F E K S G M -POI-G S A D Y K D D D D K *
IBA65:
ATGGCTAGCGATTACAAGGATGACGACGATAAGTCCGGAATG-GOI-GGGAGCTAA
M A S D Y K D D D D K S G M -POI-G S *
IBA102:
ATGAAAAAGACA-OMPA-GCGCAGGCCGCAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGT
M K K T -OMPA-A Q A A M -POI-G S A W S H P Q F E K G G S G G G S
TCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
S A W S H P Q F E K *
IBA103:
AATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
M -POI-G S A W S H P Q F E K G G G S G G G S A W S H P Q F E K *
IBA104:
ATGAAAAAGACA-OMPA-GCGCAGGCCGCAATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCG
M K K T -OMPA-A Q A A M A S A W S H P Q F E K G G G S G G G S G G S A
TGGAGCCACCCGCAGTTCGAAAAATCCGGAATG-GOI-GGGAGCTAA
W S H P Q F E K S G M -POI-G S *
IBA105:
ATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAA
M A S A W S H P Q F E K G G G S G G G S A W S H P Q F E
AAATCCGGAATG-GOI-GGGAGCTAA
K S G M -POI-G S *


IBA123:
ATGTCCCCTATA-GST-CCTCCAAAAATGTCCGGAGGTGGCGGTGGGAGCCTGGAAGTTCTGTTCCAGGGGCCAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAG
M S P I -GST-P P K M S G G G G G S L E V L F Q G P M -POI-G S A W S H P Q
TTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
F E K G G G S G G G S G G S A W S H P Q F E K *
IBA142:
ATGAGGGCCTGG-BM40-GCTCTGGCAGCAATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAA
M R A W -BM40-A L A A M A S H H H H H H S G M -POI-G S A W S H P Q F E K
GGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
G G G S G G G S G G S A W S H P Q F E K *
IBA143:
ATGGCTAGCCATCACCATCACCATCACTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGT
M A S H H H H H H S G M -POI-G S A W S H P Q F E K G G G S G G G S G G
TCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
S A W S H P Q F E K *
IBA144:
ATGAAAAAGACA-OMPA-GCGCAGGCCGCAATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCG
M K K T -OMPA-A Q A A M A S A W S H P Q F E K G G G S G G G S G G G S A
TGGAGCCACCCGCAGTTCGAAAAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
W S H P Q F E K S G M -POI-G S A H H H H H H *
IBA145:
ATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAA
M A S A W S H P Q F E K G G G S G G G S G G S A W S H P Q F E
AAATCCGGAATG-GOI-GGGAGCGCTCACCATCACCATCACCATTAA
K S G M -POI-G S A H H H H H H *
IBA162:
ATGGCTAGCGATTACAAGGATGACGACGATAAGTCCGGAATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCG
M A S D Y K D D D D K S G M -POI-G S A W S H P Q F E K G G G S G G G S
GGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAATAA
G G S A W S H P Q F E K *
IBA164:
ATGGCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAAAAATCCGGAATG-GOI-
M A S A W S H P Q F E K G G G S G G G S G G G S A W S H P Q F E K S G M -POI-
GGGAGCGCTGATTACAAGGATGACGACGATAAGTAA
G S A D Y K D D D D K *

```
IBA167:  ATGGCTAGCGATTACAAGGATGACGACGATAAGGGTGCATCTAGCGCATGGAGTCATCCTCAATTCGAGAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCG
           M  A  S  D  Y  K  D  D  D  D  K  G  A  S  S  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  G  S  G  S  A

                 TGGAGCCACCCGCAGTTCGAAAAATCCGGAATG-GOI-GGGAGCTAA
                 W  S  H  P  Q  f  E  K  S  G  M  -POI-G  S  *

IBA168:  AATG-GOI-GGGAGCGCTTGGAGCCACCCGCAGTTCGAAAAAGGTGGAGGTTCTGGCGGTGGATCGGGAGGTTCAGCGTGGAGCCACCCGCAGTTCGAGAAAGGT
           M  -POI-G  S  A  W  S  H  P  Q  F  E  K  G  G  G  S  G  G  G  S  G  G  G  S  A  W  S  H  P  Q  F  E  K  G

              GCTTCTGGTGAAGATTACAAGGATGACGACGATAAGTAA
              A  S  G  E  D  Y  K  D  D  D  D  K  *
```

7 StarGate® intergenic region cassettes

```
SD11:     AATG-GOI1-GGGAGCTAACGAGGGCAAAAAATG-GOI2-GGGA
           M  -POI1-G  S  *     RBS      M  -POI2-G

LINK11:   AATG-GOI1-GGGAGCGGCGGTGGCTCTGGTGGCGGTTCAATG-GOI2-GGGA
           M  -POI1-G  S  G  G  G  S  G  G  G  S  M  -POI2-G

LINK12:   AATG-GOI1-GGGAGCGGCGGTTCTGGTGGCGGTGGCAGCGGTGGCGGTGGCTCAATG-GOI2-GGGA
           M  -POI1-G  S  G  G  S  G  G  G  G  S  G  G  G  G  S  M  -POI2-G


IRES11:   AATG-GOI1-GGGAGCTAAGGG//IRES//ATGATAAAATG-GOI2-GGGA
           M  -POI1-G  S  *                    M  -POI2-G
```

IRES:

cgaattaattccggttattttccaccatattgccgtcttttggcaatgtgagggcccggaaacctggccctgtcttcttgacgagcattcctaggggtctttcccctctcgccaaaggaatgcaaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaa
gacaaacaacgtctgtagcgacccttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtcaaatggctctcctc
aagcgtattcaacaaggggctgaaggatgcccagaaggtaccccattgtatgggatctgatctggggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaaacgtctaggccccccgaaccacggggacgtggttttcctttgaaaaacacg

GOI - gene of interest
POI - protein of interest
OMPA - OmpA, outer membrane protein A signal sequence
GST - Glutathion-S-transferase
BM40 - basement-membrane protein 40 secretion signal sequence

The terminating part of the designation of pNFUSE-IBA vectors specifies the intergenic region by which GOI1 is linked to GOI2. The resulting gene fusion fragment as it is cloned in the Donor Vector for transfer into the Acceptor Vectors via the terminal combinatorial sites AATG and GGGA is shown above.

8 References

For up-to-date references see www.iba-lifesciences.com/StarGate_Cloning.html

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