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StarGate®

The new dimension of combinatorial cloning

Instruction manual

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Content

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, we have developed the StarGate® system. StarGate® offers rapid and highly efficient subcloning of an arbitrary gene – initially cloned in a Donor Vector - to fuse it in parallel with several different genetic surroundings via transfer into Acceptor Vectors to generate Destination Vectors (cf. section 5.1).

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 List of necessary components

2.1 Donor Vector generation containing wild type or mutated GOI

2.1.1 StarGate® Combi Entry Cloning

StarGate® Combi Entry Cloning Set; 20 rxn	**Cat.No. 5-1608-000**

consisting of:

StarGate® Combi Entry Reagent Set; 20 rxn	Cat.No. 5-1608-001
20 rxn	pENTRY-IBA51 (10 µl each)
23 µl	StarSolution M1
23 µl	StarSolution M2
11.5 µl	StarSolution M3, has to be diluted with M3-Diluent prior to use: Add 11.5 µl M3-Diluent, mix gently but thoroughly and spin briefly prior to use to recover contents. Mark vessel with a "+" after addition of M3-Diluent.
15 µl	M3-Diluent
45 µl	ENTRY-Primer-for2 (10 µM)
45 µl	ENTRY-Primer-rev (10 µM)
100 µl	DNA ruler (manufactured by Fermentas)
Storage	-18 °C to -20 °C (long term, do not use frost free freezers); on ice (during use)
Comments	Spin reagents briefly after thawing and mixing prior to use to recover all contents.

20 rxn	Competent cells *E. coli* Top10 (100 µl each)	Cat.No. 5-1600-020
Storage	- 80 °C	
Comments	Avoid any unnecessary warming of the cells by repacking or during storage until transformation. Even warming without thawing may already decrease ability of the cells for plasmid DNA uptake. Do not spin or vortex prior to use.	

Additional materials required	• Primer set for amplification of the gene of interest and attachment of combinatorial sites • *Pfu* DNA polymerase and corresponding PCR reagents • 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

2.1.2 StarGate® Fusion Cloning

StarGate Fusion Cloning Sets; 5 rxn	
StarGate® Fusion Cloning Set "IRES1"	Cat.No. 5-1607-001
StarGate® Fusion Cloning Set "SD1"	Cat.No. 5-1607-002
StarGate® Fusion Cloning Set "LINK1"	Cat.No. 5-1607-003
StarGate® Fusion Cloning Set "LINK2"	Cat.No. 5-1607-004

consisting of:

respective pNFUSE-IBA vector		
5 rxn	pNFUSE-IBA-IRES11 (10 µl each)	Cat.No. 5-1631-005
5 rxn	pNFUSE-IBA-SD11 (10 µl each)	Cat.No. 5-1632-005
5 rxn	pNFUSE-IBA-LINK11 (10 µl each)	Cat.No. 5-1633-005
5 rxn	pNFUSE-IBA-LINK12 (10 µl each)	Cat.No. 5-1634-005
Storage	-18°C to -20°C (long term, do not use frost free freezers); on ice (during use)	
Comments	Spin reagents briefly after thawing and mixing prior to use to recover all contents.	

components of each Set in addition:

StarGate Fusion Reagent Set; 5 rxn	Cat.No. 5-1607-000
5 rxn	pCFUSE-IBA11 (10 µl each)
5 rxn	pENTRY-IBA51 (10 µl each)
12 µl	StarSolution F1
12 µl	StarSolution F2
5 µl	StarSolution F3, has to be diluted with F3-Diluent prior to use: Add 6.5 µl F3-Diluent, mix gently but thoroughly and spin briefly prior to use to recover contents. Mark vessel with a “+” after addition of F3-Diluent.
20 µl	F3-Diluent
7.5 µl	StarSolution F4
7.5 µl	StarSolution F5
4 µl	StarSolution F6, has to be diluted with F6-Diluent prior to use: Add 4 µl F6-Diluent, mix gently but thoroughly and spin briefly prior to use to recover contents. Mark vessel with a “+” after addition of F6-Diluent.
7 µl	F6-Diluent
Storage	-18°C to -20°C (long term, do not use frost free freezers); on ice (during use)
Comments	Spin reagents briefly after thawing and mixing prior to use to recover all contents.

15 rxn	Competent cells E. coli Top10 (100 µl each)	Cat. No. 5-1600-015
Storage	- 80°C	
Comments	Avoid any unnecessary warming of the cells by repacking or during storage until transformation. Even warming without thawing may already decrease ability of the cells for plasmid DNA uptake. Do not spin or vortex prior to use.	

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

2.2 Destination Vector generation

2.2.1 StarGate® Transfer Cloning

StarGate® Transfer Reagent Set; 20 rxn	**Cat.No. 5-1603-001**

consisting of:

23 µl	StarSolution A1
23 µl	StarSolution A2
10 µl	StarSolution A3, has to be diluted with A3-Diluent prior to use: Add 13 µl A3-Diluent, mix gently but thoroughly and spin briefly prior to use to recover contents. Mark vessel with a "+" after addition of A3-Diluent.
20 µl	A3-Diluent
Storage	-18 °C to -20 °C (long term, do not use frost free freezers); on ice (during use)
Comments	Spin reagents briefly after thawing and mixing prior to use to recover all contents.

5 rxn	Acceptor Vector (10 µl each) (for individual Cat.No. see table 1, p.29)
Storage	-18 °C to -20 °C (long term, do not use frost free freezers); on ice (during use)
Comments	Spin reagents briefly after thawing and mixing prior to use to recover all contents.

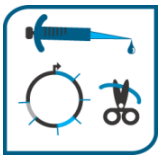
20 rxn	Competent cells *E. coli* Top10 (100 µl each)	Cat.No. 5-1600-020
Storage	- 80 °C	
Comments	Avoid any unnecessary warming of the cells by repacking or during storage until transformation. Even warming without thawing may already decrease ability of the cells for plasmid DNA uptake. Do not spin or vortex prior to use.	

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

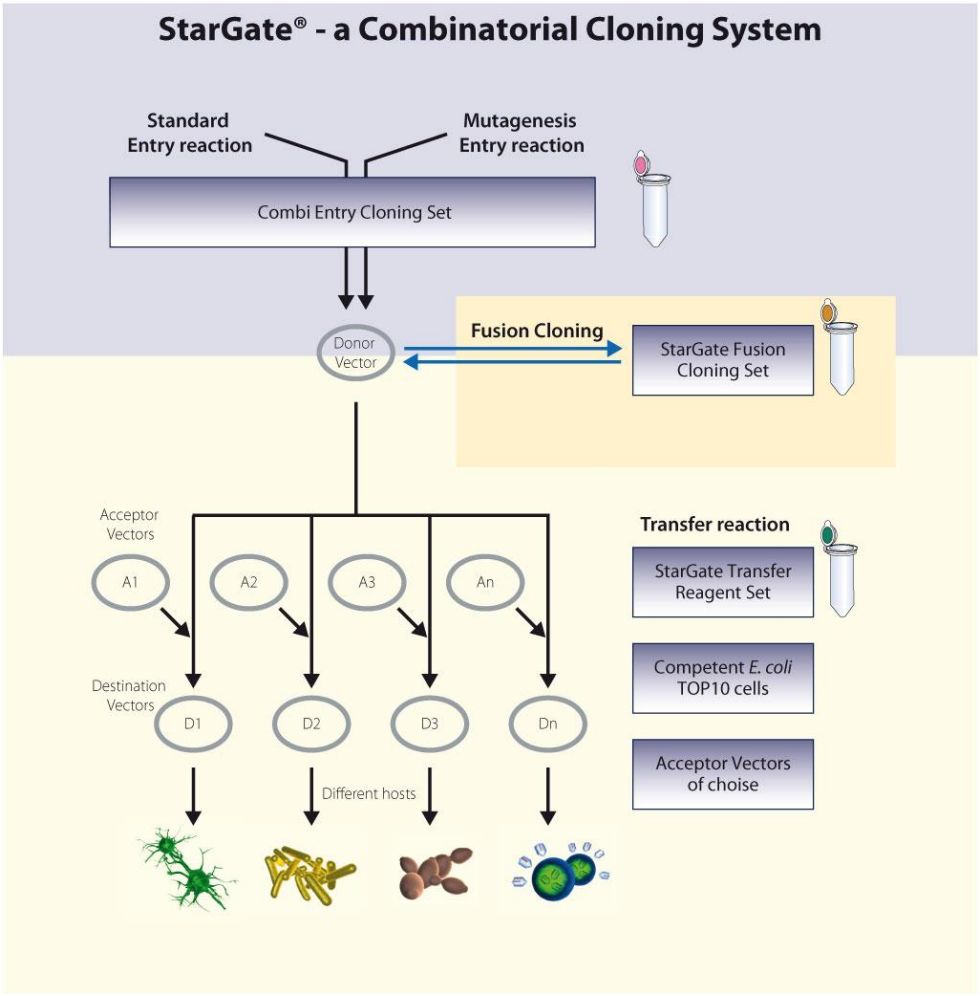
2.3 Control reactions (part of StarGate® Combi Newcomer Set, Cat.No. 5-1600-996)

25 µl	400 bp PCR-fragment (2 nM)	Cat.No. 5-1635-000
25 µl	Donor Vector with 400 bp DNA insert (2 ng/µl)	Cat.No. 5-1636-000
Storage	-18 °C to -20 °C (long term, do not use frost free freezers); on ice (during use)	
Comments	Spin reagents briefly after thawing and mixing prior to use to recover all contents.	

3 StarGate® procedure



3.1 Workflow (schematic view)



Step 1: Donor Vector generation (Combi Entry Cloning)

In the first step, the gene of interest (GOI) is extended by PCR at both ends with the combinatorial and StarCombinase™ sites (orange and red; 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 an Entry Vector by a simple one-tube reaction using a specific StarCombinase™. See Chapter 3.2 for detailed description.

Step 2: Destination Vector generation (Transfer reaction)

After sequence confirmation the resulting Donor Vector serves as basis for the optional highly parallel subcloning of GOI into a multitude of Acceptor Vectors by a second simple one-tube reaction, where each Acceptor Vector provides a different genetic surrounding like host specific promoters and different purification tags. The corresponding host cells are then

transformed with the resulting Destination Vectors. Afterwards optimal conditions for expression and purification of a given GOI may be systematically screened. See Chapter 3.3 for detailed description.

Optional intermediate step: Fusion Cloning

StarGate® fusion cloning is an intermediate step between the Entry reaction and the Transfer reaction. It allows easy and fast fusion of two genes of interest (GOI-1 and GOI-2) present in separate Donor Vectors by performing two sequential StarGate® subcloning reactions.

3.2 Step 1: Donor Vector generation

There are several possibilities to generate a Donor Vector:

1) **Wild type GOI's** are inserted into the Entry Vector pENTRY-IBA51 by using the Combi Entry Cloning Set after simple PCR amplification of GOI (please refer to 3.2.1).

2) For the expression of **mutated derivatives** of the GOI, a modified PCR process together with the same Combi Entry Cloning Set is used to generate the Donor Vector by means of pENTRY-IBA51 (please refer to 3.2.2).

3) **Fusions** between two or more GOI's can be generated starting with Donor Vectors obtained from procedures 1) or 2) above (please refer to 3.2.3 Fusion Cloning).

3.2.1 Donor Vector with wild type GOI with Combi Entry Cloning Set

In a first step, the gene of interest (GOI) has to be equipped with combinatorial sites and the StarCombinase™ recognition area at both termini which are important for oriented insertion of the PCR fragment into pENTRY-IBA51 (Figure 1).

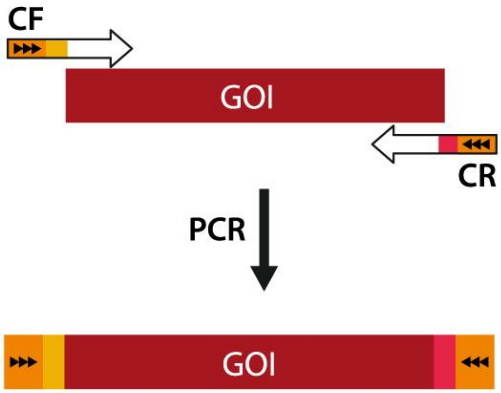


Fig. 1
The forward primer (CF) starts with the StarCombinase1™ recognition area 5'-AGCGGCTCTTC (orange with arrows indicating its orientation) followed by the upstream AATG combinatorial site, including the start codon ATG, which is further followed by nucleotides complementary to the antisense strand of GOI. The reverse primer (CR) equally starts with the StarCombinase1™ recognition area 5'-AGCGGCTCTTC followed by TCCC, the reverse complement of the downstream combinatorial site GGGA, which again is directly followed by the reverse complement of the nucleotides encoding the C-terminal amino acids of 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) and StarCombinase1™ recognition area (underlined). 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.

Important notes

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

In a second step, the PCR product is inserted into the Entry Vector pENTRY-IBA51 which thereby results in a Donor Vector (Figure 2).

This is achieved by mixing the Entry Vector with the PCR product, adding StarSolutions “M” and incubating the resulting mixture for one hour.

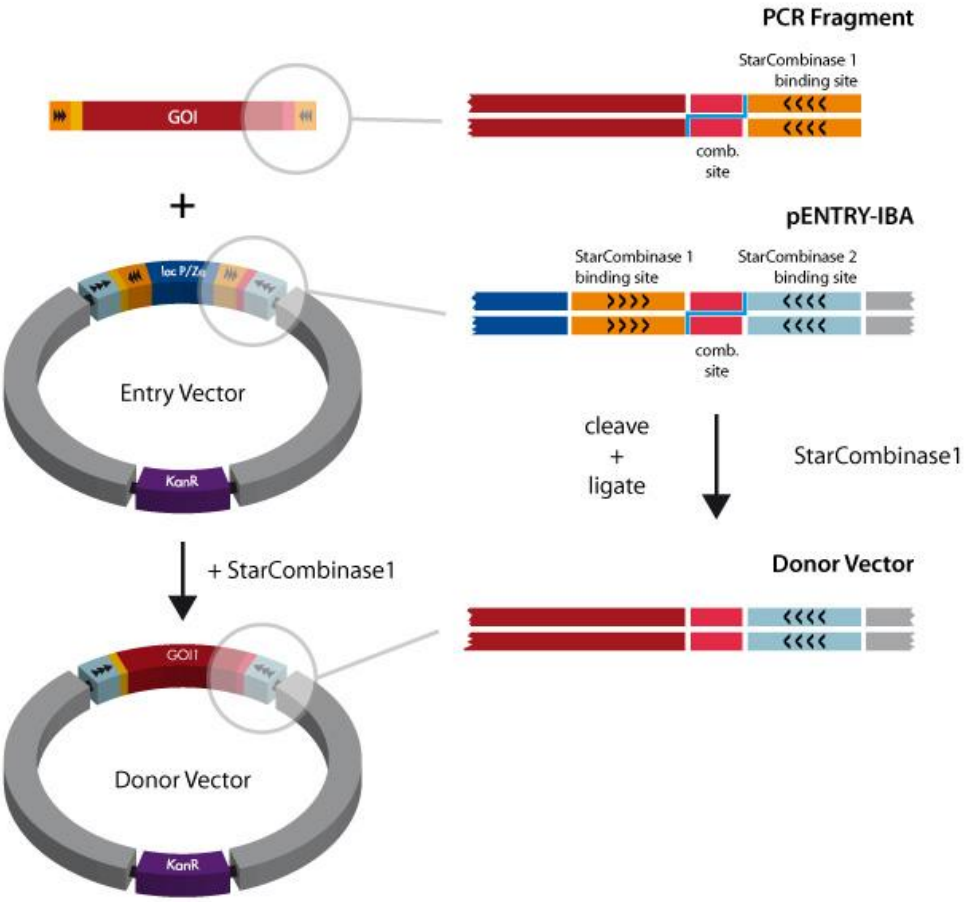


Fig. 2
Insertion of GOI into the Entry Vector pENTRY-IBA51 to create a Donor Vector.

StarCombinase™ formulations simultaneously cleave (in a shifted manner remote from the recognition area creating overhangs which are named combinatorial sites) and ligate DNA. Thus, using StarCombinase1™, the PCR product and the Entry Vector are recombined at the combinatorial sites (red and light orange) leading to the generation of the Donor Vector under loss of all StarCombinase1™ recognition areas (dark orange). This makes the recombination reaction unidirectional and thereby highly efficient. In the resulting Donor Vector the same combinatorial sites are now under control of another StarCombinase™, i.e., StarCombinase2™, thereby enabling a highly efficient and specific StarGate® gene transfer process into correspondingly designed Acceptor Vectors.

Insertion of PCR product/GOI in 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 included in the kit.

3.2.2 Mutated GOI with Combi Entry Cloning Set

To generate a StarGate® compatible Donor Vector with a modified sequence of the GOI, StarGate® offers a special PCR-based procedure. The design of appropriate PCR primers is simplified by the free software StarPrimer D'Signer (Please note: If you have problems with downloading the StarPrimer D´Signer software, either send an e-mail to stargate@iba-lifesciences.com or call +49 551-50672-0, we will be glad to assist you).

For this purpose, the sequences of the initial, wild type gene of interest (as it is present in the template DNA for PCR) as well as the sequence of the desired mutated gene have both to be copied, without start and stop codon, and pasted into the corresponding menu field of the program. Dependent on the site where the mutation(s) have to be introduced, 2 or 4 primers are designed by the program.

<u>Case 1: Internal mutations</u>

In this case, 4 primers are designed. The 2 primers annealing at the extreme ends of GOI are identical with those for cloning wild type GOI in pENTRY-IBA51 (CF and CR) while the internal primer pair is new and introduces the mutation(s) (MF and MR).

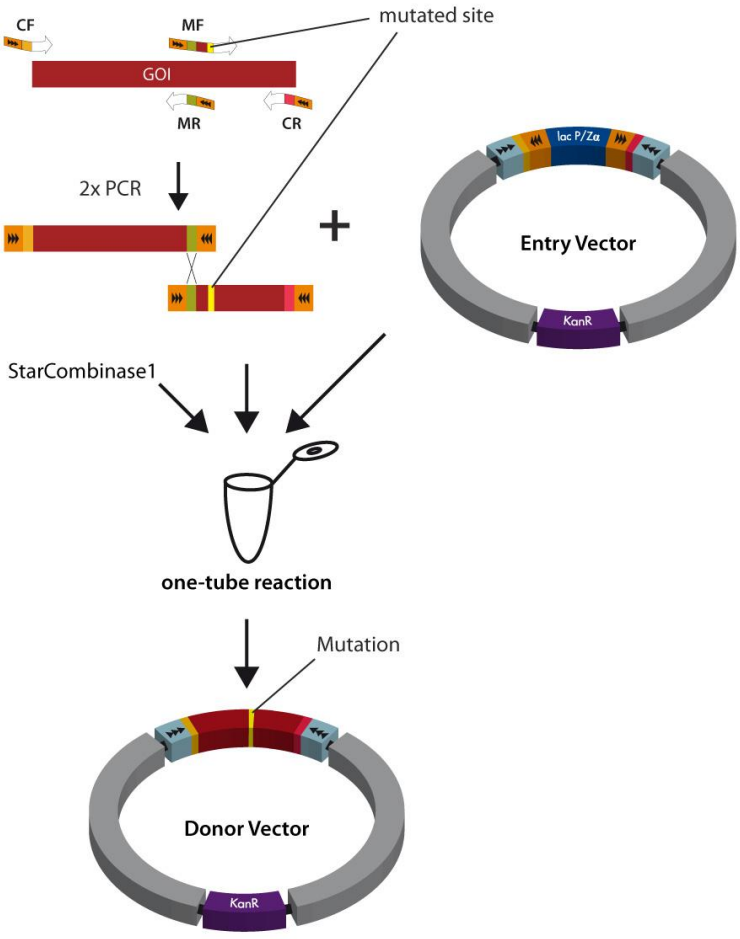


Fig. 3A
Directed assembly of 2 PCR products in pENTRY-IBA51 to create a Donor Vector with a mutated GOI.

Case 2: Mutations at the extreme ends

In this case, 2 primers are sufficient by using an elongated primer containing the desired mutation(s) at the corresponding site. N-terminal mutations are introduced by a primer denoted CMF (Figure 3B) and C-terminal mutations are introduced by a primer denoted CMR (Figure 3C) while the respective opposite primer is the same as for cloning a wild type GOI (CR or CF).

Fig. 3B: N-terminal mutations

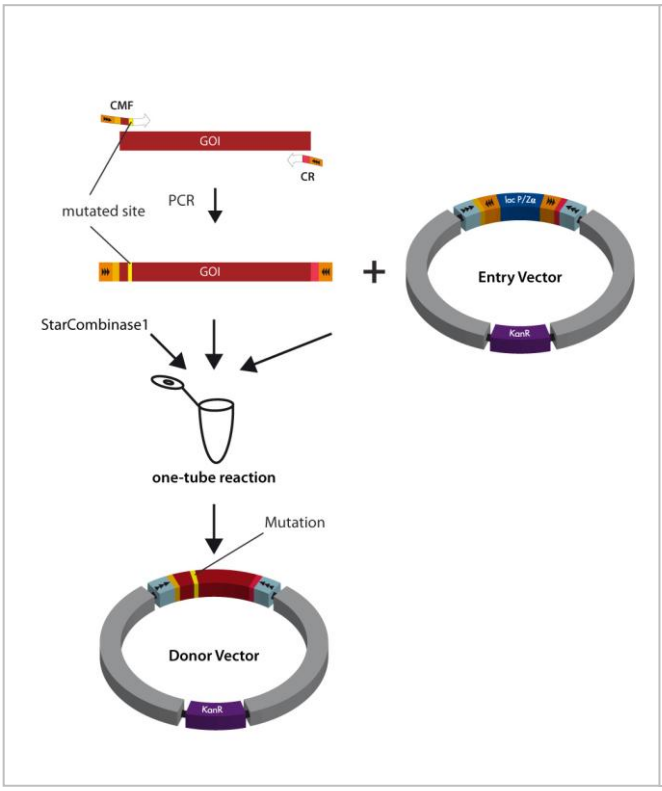
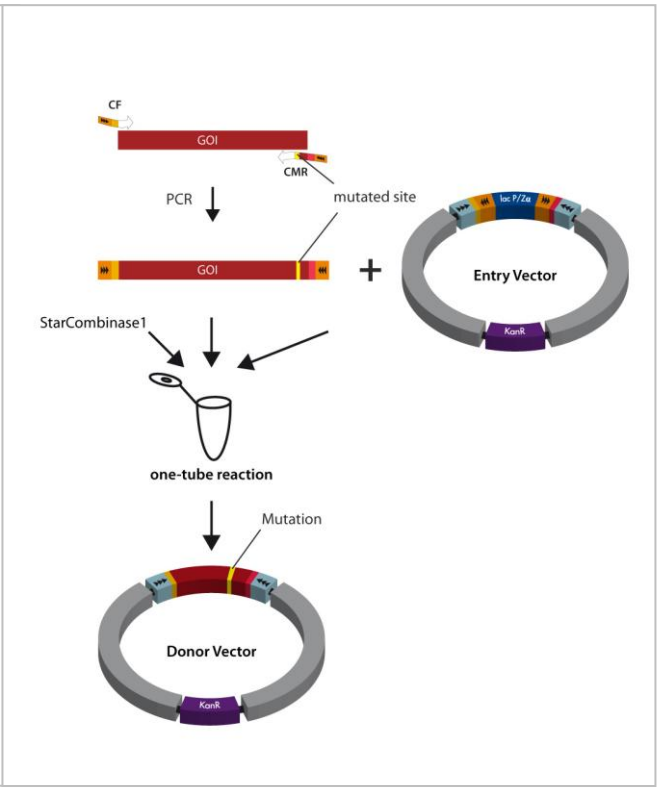


Fig. 3C: C-terminal mutations



In all cases, the PCR product(s) are assembled with the Entry Vector pENTRY-IBA51 by StarCombinase1™ in a directed manner.

Important notes

- Use a proof reading DNA polymerase like *Pfu* (Fermentas) that minimizes the risk of mutations.
- Use 3' phosphorothioate (PTO) protected primers in case of using a proof reading DNA polymerase.
- Insertion of PCR product(s) can be checked by restriction analysis.
- As PCR may lead to non-desired mutations, it is recommended to confirm mutated GOI sequence by Donor Vector sequencing using ENTRY-Primer-for2 and/or ENTRY-Primer-rev included in the kit.

3.2.3 Fusion of two or more GOI's with StarGate® Fusion Cloning Set

Two or more genes that are already cloned into separate Donor Vectors can be connected by an intergenic region (IR). The IR may e.g., code for an amino acid linker sequence directly connecting the gene product of a first GOI with the product of a second GOI. The IR may also include a Shine Dalgarno (prokaryotes) or IRES (eukaryotes) site for the construction of synthetic operons or promoters for the completely independent expression of different GOIs from one vector.

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

In the first reaction, the two GOIs to be fused are 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 construction is transferred into a pNFUSE-IBA derivative specifying the IR, while the GOI to be positioned downstream 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 in pENTRY-IBA51 by a second one-tube reaction. An overview of fusion cloning is given by Figure 4A and, including details, by Figure 4B.

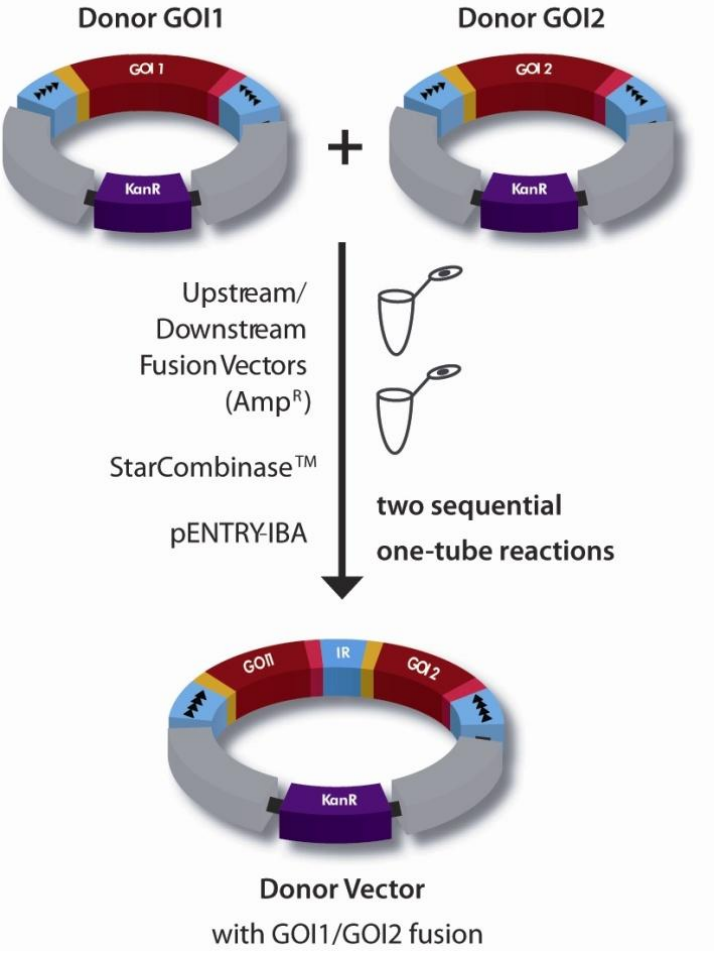


Fig. 4A
Two GOI's, GOI1 and GOI2, are fused by an intergenic region denoted by "IR".

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 under 4B).

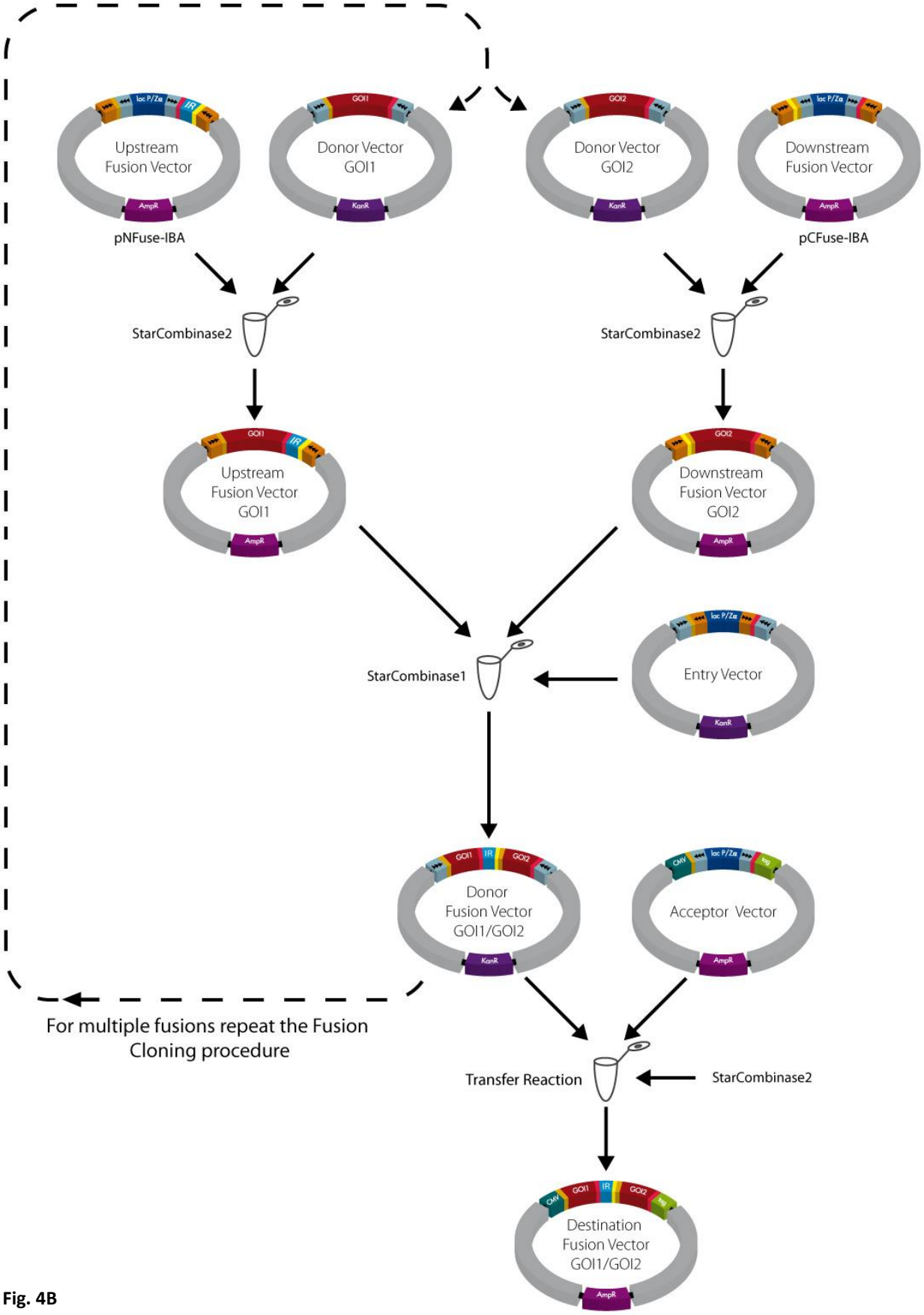


Fig. 4B
Detailed overview of StarGate® fusion cloning

3.3 Step 2: 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 here the different genetic surroundings (i.e., tag, promoter, signal sequence etc., see table 1, page 29) for the expression vector and are mixed with the Donor Vector and Star Solutions A1-A3 forming in a second one-tube reaction the Destination Vector containing the GOI (Figure 5).

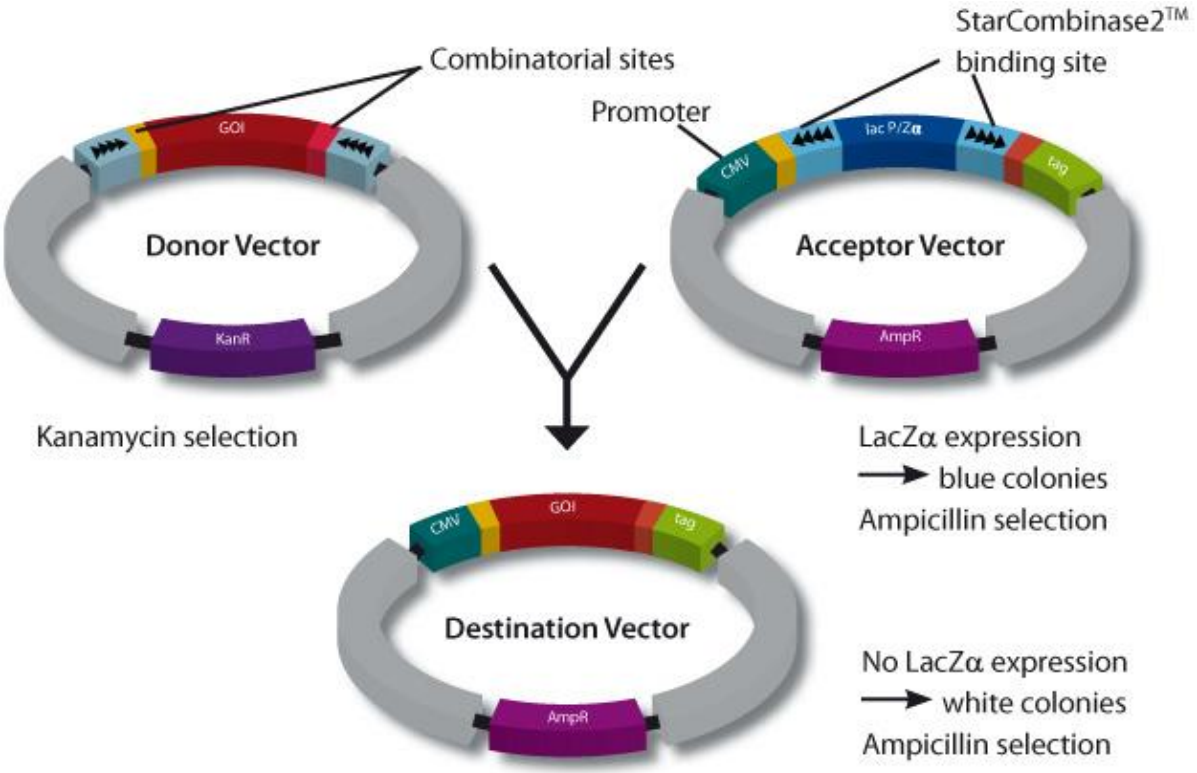


Fig. 5
Recombination will take place at the AATG and GGGA combinatorial sites, thereby imposing the occurrence of these sites in the final Destination Vector. The more complex recognition sites are eliminated and not expressed. Loss of the recognition sites drives the reaction towards generation of the 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 non-desired Acceptor Vectors will generate blue colonies (Figure 6, page 17).

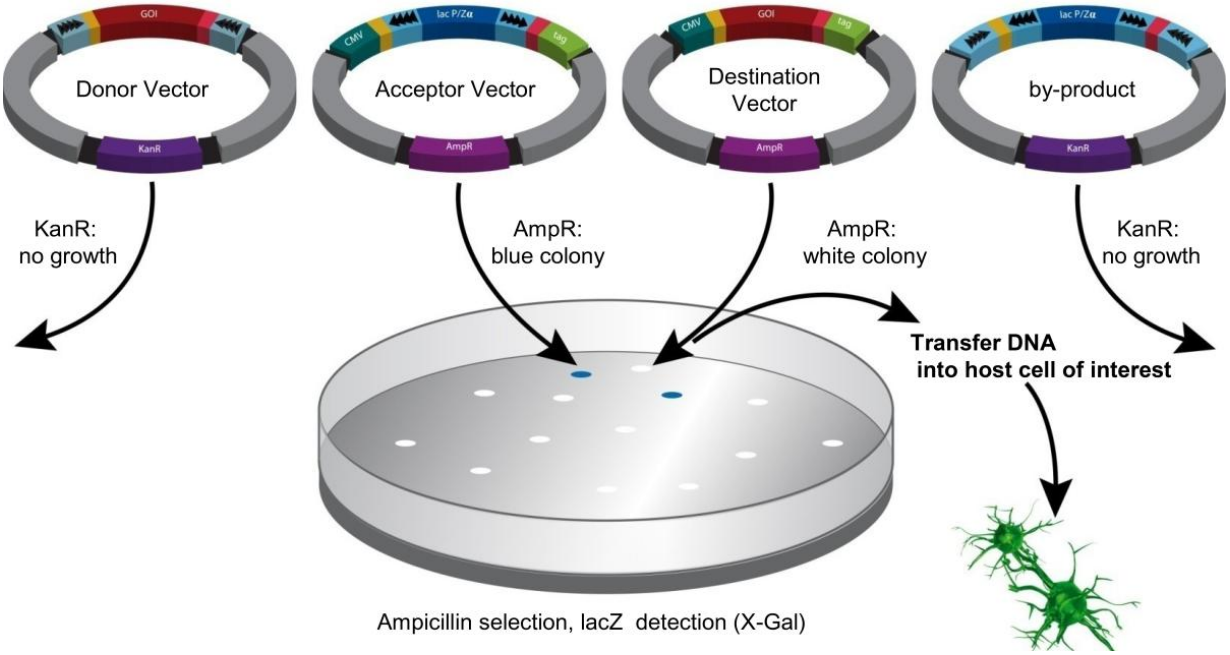


Fig. 6
E. coli is transformed with the mixture that potentially includes all 4 possible vectors. Due to selection on ampicillin plates, Donor Vector and by product – which provide a kanamycin resistance gene only – will not enable growth of *E. coli*. Acceptor Vector and Destination Vectors, however, enable growth due to the encoded ampicillin resistance genes. The Acceptor Vector carries the LacZα gene and, therefore, produces blue colonies on X-gal containing plates while LacZα has been replaced by GOI in the Destination Vector which, therefore, generates white colonies. The Destination Vector of this example places the GOI under control of the CMV promoter enabling GOI expression in mammalian cells and fuses a tag to the C-terminal end of GOI expression product.

4 StarGate® protocols/recommendations

4.1 Step 1: Donor Vector generation with Combi Entry Cloning Set for wild type GOI

4.1.1 PCR to amplify and equip GOI with AATG and GGGA combinatorial and StarCombinase™ sites

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 and consequently yield of 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 Primer 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’-AGCGGCTCTTC***AATG***TTGACCTGCAACAGCTGCAT-3’
                  ||||||||||||||||||||
               5’-TTGACCTGCAACAGCTGCATAGCC-3’
               3’-AACTGGACGTTGTCGACGTATCGG-5’
                       ||||||||||||||||||||
                    3’-GACGTTGTCGACGTATCGG***CCCT***CTTCTCGGCGA-5’
```

(CR: reverse primer)

5’ end of the CF primer (forward primer) is elongated by the AATG combinatorial site (italic and bold) and the StarCombinase1™ recognition area (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 StarCombinase1™ recognition area (underlined).

Continue page 19

Important notes, continued

The resulting PCR product then has the following structure:

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

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 in a total volume of 50µl** (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	30s - 1 min	Annealing	
72 °C	30s - 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 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.).

Apply 5 - 10 µl DNA Ruler per lane.

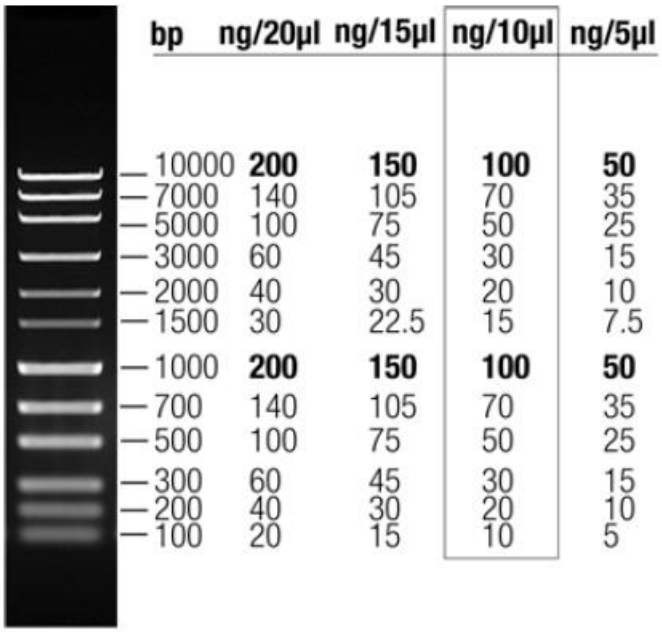


Fig. 7
DNA Ruler is a molecular size standard where each band represents a defined amount of linear DNA.

4.1.2 Reaction for Donor Vector generation (wild type GOI)

Protocol

1. **Add the following reagents to the supplied reaction tube containing 10 µl of the Entry Vector pENTRY-IBA51:**

12 µl	PCR product (2 nM) from 4.1.1.2
1 µl	StarSolution M1
1 µl	StarSolution M2
1 µl	StarSolution M3 (supplemented with M3-Diluent, see 2.1.1)

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

4.1.3 Donor Vector identification (wild type GOI)

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 XbaI/HindIII restriction sites and consider the changed DNA fragment sizes resulting thereof).

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 included in the kit (ENTRY-Primer-for2 and ENTRY-Primer-rev). 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 Step 1: Donor Vector generation with Combi Entry Cloning Set for mutated GOI

4.2.1 PCRs to create the mutated GOI sequence

Important notes

Primer design is conveniently achieved by the StarPrimer D'Signer software. Typically, the software will determine 4 primers, i.e.,
CF (forward primer; identical to the forward primer for cloning the wt GOI),
MR (mutagenesis reverse primer),
MF (mutagenesis forward primer) and
CR (reverse primer) (please refer to Figure 3A).
In exceptional cases – if mutations to be introduced are close to an extreme end of GOI – 2 primers will be determined only, i.e., CMF (mutagenesis forward primer) or CMR (mutagenesis reverse primer). In this case, the non-mutagenic opposite primer corresponds to the respective standard primer CF or CR (please refer to Figure 3B and 3C).

Protocol

1. **Amplify the GOI by two separate PCR's using the primer pairs CF/MR and MF/CR (or alternatively by one PCR with CF/CMR or CMF/CR).**
   In principle, also mutagenic end primers (CMF and/or CMR) can be combined with a mutagenic internal primer pair (MF/MR). Perform PCR and determine PCR product concentration as described in paragraph 4.1.1.

2. **In case of two PCR products (C(M)F/MR and MF/C(M)R), dilute each PCR product to 4 nM with water** (corresponds to 1.4 ng/µl for a 0.5 kb fragment, 2.8 ng/µl for a 1 kb fragment, 4.2 ng/µl for a 1.5 kb fragment, 5.6 ng/µl for a 2 kb fragment, 7 ng/µl for a 2.5 kb fragment, etc.).

3. **In case of one PCR product (CF/CMR or CMF/CR or CMF/CMR), 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.).

4.2.2 Reaction for Donor Vector generation (mutated GOI)

Protocol

1. **Add the following reagents to the supplied reaction tube containing 10 µl of the Entry Vector pENTRY-IBA51:**

6 µl*	Water diluted PCR product (CF/MR, 4 nM) from 4.2.1
6 µl*	Water diluted PCR product (MF/CR, 4 nM) from 4.2.1
1 µl	StarSolution M1
1 µl	StarSolution M2
1 µl	StarSolution M3 (supplemented with M3-Diluent, see 2.1.1)

*When only 1 PCR product has to be prepared (cf. 4.2.1), use 12 µl of the 2 nM solution of this PCR product instead of 2x 6 µl.

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.**

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Protocol (continued)

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 plates as above.**

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

4.2.3 Donor Vector identification (mutated GOI)

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 having the length of the PCR product from 4.2.1 plus 40 bases is expected as long as no *Xba*I and/or *Hin*dIII restriction sites are present internally in GOI.

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 included in the kit (ENTRY-Primer-for2 and ENTRY-Primer-rev). The region flanking the mutated GOI should have the sequence:

   ```
   TCTAGAAAAGCGCGTCTCC***AATG***-mutGOI-***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.**

4.3 Optional intermediate step: Donor vector generation with fused GOI's from separate Donor Vectors with StarGate® Fusion Cloning Set

4.3.1 Transfer of GOI's into Fusion Vectors

Important notes

The GOI to be positioned upstream in the final fusion has to be transferred into the pNFUSE-IBA derivative carrying the desired intergenic region (IR; please refer to chapter 1 for IR sequences). The GOI to be positioned downstream in the final fusion 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. **Add the reagents below to the supplied reaction tube containing 10 µl of the appropriate Fusion Vector**

12 µl	Diluted Donor Vector solution (2 ng/µl; see 4.1.3 or 4.2.3)
1 µl	StarSolution F1
1 µl	StarSolution F2
1 µl	StarSolution F3 (supplemented with F3-Diluent, see2.1.2)

2. **Close the reaction vessel thoroughly, mix gently and incubate at 30 °C for 1 h.**
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. **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.**
9. **Incubate plates over night at 37 °C.**

4.3.2 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 XbaI/HindIII restriction sites and consider the changed DNA fragment sizes resulting thereof.

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.**

4.3.3 Assembly of GOI1 and GOI2 in pENTRY-IBA51

Protocol

1. **Add the following reagents to the supplied reaction tube containing 10 µl of the Entry Vector pENTRY-IBA51:**

6 µl	Diluted upstream Fusion Vector with GOI1 (4 ng/µl) from 4.3.2
6 µl	Diluted downstream Fusion Vector with GOI2 (4 ng/µl) from 4.3.2
1 µl	StarSolution F4
1 µl	StarSolution F5
1 µl	StarSolution F6 (supplemented with F6-Diluent, see 2.1.2)

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, ad 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.

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Protocol (continued)

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

4.3.4 Donor Vector identification (fused GOI1 + GOI2)

Protocol

1. **Pick 5 white colonies and perform DNA mini preparation.**
2. **Perform *Xba*I/*Hin*dIII restriction.**
   A fragment with the length of mutated GOI plus 40 bases is expected (Check your GOI for internal XbaI/HindIII restriction sites and consider the changed DNA fragment sizes resulting thereof).
3. **Select a putatively correct clone and optionally confirm sequence via Donor Vector forward and reverse sequencing primers (ENTRY-Primer-for2 and ENTRY-Primer-rev).**
   The combinatorial site flanking region should have the sequence:

```
TCTAGAAAAGCGCGTCTCC***AATG***-GOI1-***GGGA***-IR-***AATG***-GOI2-***GGGA***GGAGACGCGCTAAAAGCTT
 XbaI                                                                    HindIII
```

   Possible intergenic region (IR) sequences can be found in chapter 1.
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.**

4.4 Step 2: Destination Vector generation

4.4.1 GOI transfer reaction

Protocol

1. Add the reagents below to the supplied reaction tube containing 10 µl of the appropriate Acceptor Vector:

12 µl	Diluted Donor Vector solution (2 ng/µl; see 4.1.3 or 4.2.3 or 4.3.4)
1 µl	StarSolution A1
1 µl	StarSolution A2
1 µl	StarSolution A3 (supplemented with A3-Diluent, see 2.2.1)

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

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

4. After incubation, pipet off an aliquot of 10 µl 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.

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. 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.

9. Incubate plates over night at 37 °C.

4.4.2 Destination Vector identification

Protocol

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

2. pASG-IBA, pPSG-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

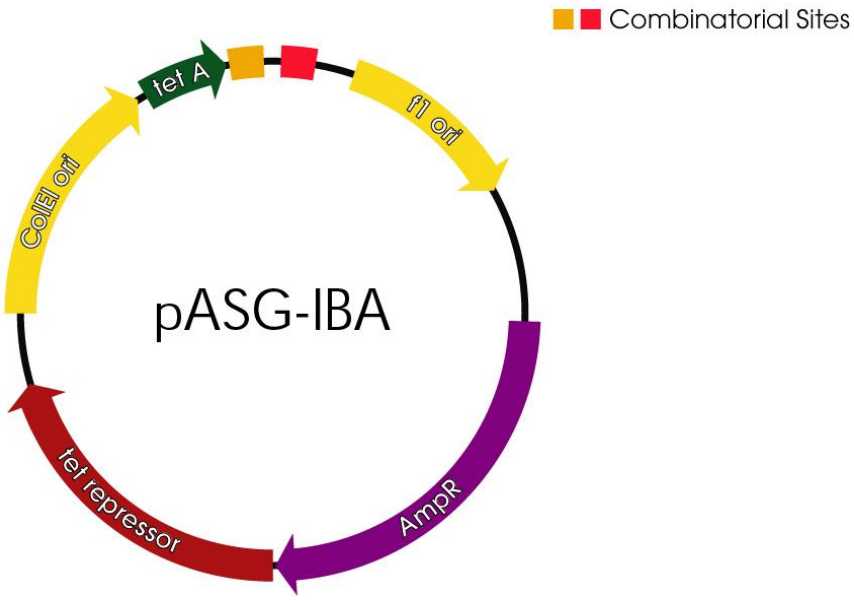
5.1 Overview

pASG-	pPSG-	pESG-	pCSG-	pLSG-	pYSG-		N-terminal					C-terminal
									CS	GOI	CS	
5-4000-000	5-4200-000	5-4400-000	5-5000-000	5-4800-000	5-4600-000	IBAwt1			CS	GOI	CS	
5-4005-000	5-4205-000	5-4405-000	5-5005-000	5-4805-000	5-4605-000	IBA5		*Strep*	CS	GOI	CS	
5-4105-000	5-4305-000	5-4505-000	5-5105-000	5-4905-000	5-4705-000	IBA105		One-STrEP	CS	GOI	CS	
5-4035-000	5-4235-000	5-4435-000	5-5035-000	5-4835-000	5-4635-000	IBA35		6xHistidine	CS	GOI	CS	
5-4025-000	5-4225-000	5-4425-000	5-5025-000	5-4825-000	5-4625-000	IBA25		GST	CS	GOI	CS	
5-4003-000	5-4203-000	5-4403-000	5-5003-000	5-4803-000	5-4603-000	IBA3			CS	GOI	CS	*Strep*
5-4103-000	5-4303-000	5-4503-000	5-5103-000	5-4903-000	5-4703-000	IBA103			CS	GOI	CS	One-STrEP
5-4033-000	5-4233-000	5-4433-000	5-5033-000	5-4833-000	5-4633-000	IBA33			CS	GOI	CS	6xHistidine
5-4045-000	5-4245-000	5-4445-000	5-5045-000	5-4845-000	5-4645-000	IBA45		*Strep*	CS	GOI	CS	6xHistidine
5-4145-000	5-4345-000	5-4545-000	5-5145-000	5-4945-000	5-4745-000	IBA145		One-STrEP	CS	GOI	CS	6xHistidine
5-4043-000	5-4243-000	5-4443-000	5-5043-000	5-4843-000	5-4643-000	IBA43		6xHistidine	CS	GOI	CS	*Strep*
5-4143-000	5-4343-000	5-4543-000	5-5143-000	5-4943-000	5-4743-000	IBA143		6xHistidine	CS	GOI	CS	One-STrEP
5-4023-000	5-4223-000	5-4423-000	5-5023-000	5-4823-000	5-4623-000	IBA23		GST	CS	GOI	CS	*Strep*
5-4123-000	5-4323-000	5-4523-000	5-5123-000	5-4923-000	5-4723-000	IBA123		GST	CS	GOI	CS	One-STrEP
5-4065-000	5-4265-000	5-4465-000	5-5065-000	5-4865-000	5-4665-000	IBA65		FLAG	CS	GOI	CS	
5-4063-000	5-4263-000	5-4463-000	5-5063-000	5-4863-000	5-4663-000	IBA63			CS	GOI	CS	FLAG
5-4062-000	5-4262-000	5-4462-000	5-5062-000	5-4862-000	5-4662-000	IBA62		FLAG	CS	GOI	CS	*Strep*
5-4162-000	5-4362-000	5-4562-000	5-5162-000	5-4962-000	5-4762-000	IBA162		FLAG	CS	GOI	CS	One-STrEP
5-4064-000	5-4264-000	5-4464-000	5-5064-000	5-4864-000	5-4664-000	IBA64		*Strep*	CS	GOI	CS	FLAG
5-4164-000	5-4364-000	5-4564-000	5-5164-000	5-4964-000	5-4764-000	IBA164		One-STrEP	CS	GOI	CS	FLAG
5-4167-000	5-4367-000	5-4567-000	5-5167-000	5-4967-000	5-4767-000	IBA167	FLAG	One-STrEP	CS	GOI	CS	
5-4168-000	5-4368-000	5-4568-000	5-5168-000	5-4968-000	5-4768-000	IBA168			CS	GOI	CS	One-STrEP
5-4001-000		5-4401-000	5-5001-000	5-4801-000		IBAwt2			CS	GOI	CS	
5-4004-000						IBA4		*Strep*	CS	GOI	CS	
5-4104-000		5-4504-000	5-5104-000	5-4904-000		IBA104		One-STrEP	CS	GOI	CS	
5-4002-000						IBA2			CS	GOI	CS	*Strep*
5-4102-000		5-4502-000	5-5102-000	5-4942-000		IBA102			CS	GOI	CS	One-STrEP
5-4044-000						IBA44		*Strep*	CS	GOI	CS	6xHistidine
5-4144-000		5-4544-000	5-5144-000	5-4944-000		IBA144		One-STrEP	CS	GOI	CS	6xHistidine
		5-4542-000	5-5142-000	5-4902-000		IBA142		6xHistidine	CS	GOI	CS	One-STrEP
OmpA		BM40	BM40	BM40								

CS=combinatorial site (AATG and GGGA);
GOI=gene of interest

Table 1: The first part of the vector name specifies the expression system while the second part specifies the expression cassette. StarGate® Acceptor Vector collection will be constantly expanded. Please inquire at info@iba-lifesciences.com when a suitable Acceptor Vector for the desired application cannot be found as this table may not represent the most recent state. IBA also offers a custom service for adaptation of your favorite expression vector to StarGate®. Please inquire.

5.2 pASG-IBA



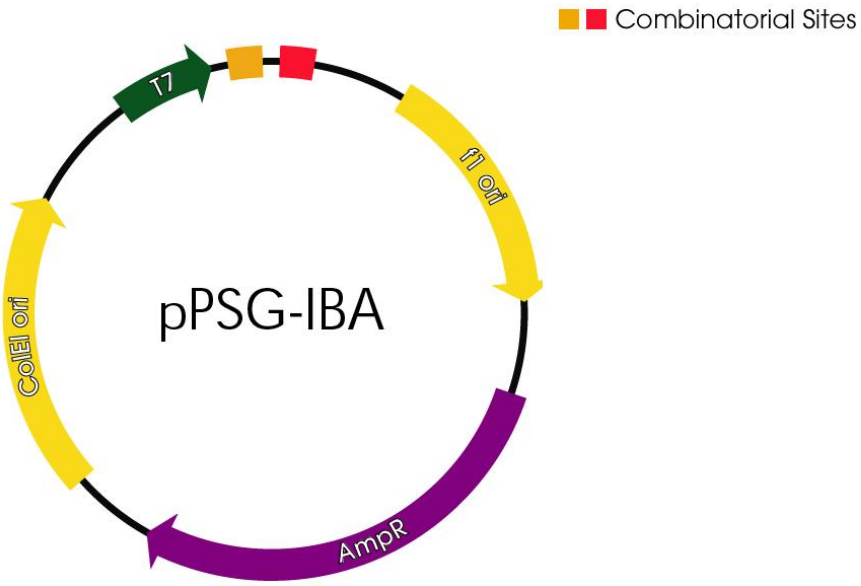
The production of a heterologous protein is often accompanied by an impaired growth of *E. coli* cells. Consequently, regulation of heterologous biosynthesis is generally recommended by the use of a promoter whose activity can be blocked by a repressor. If the foreign protein is cytotoxic, even the production 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



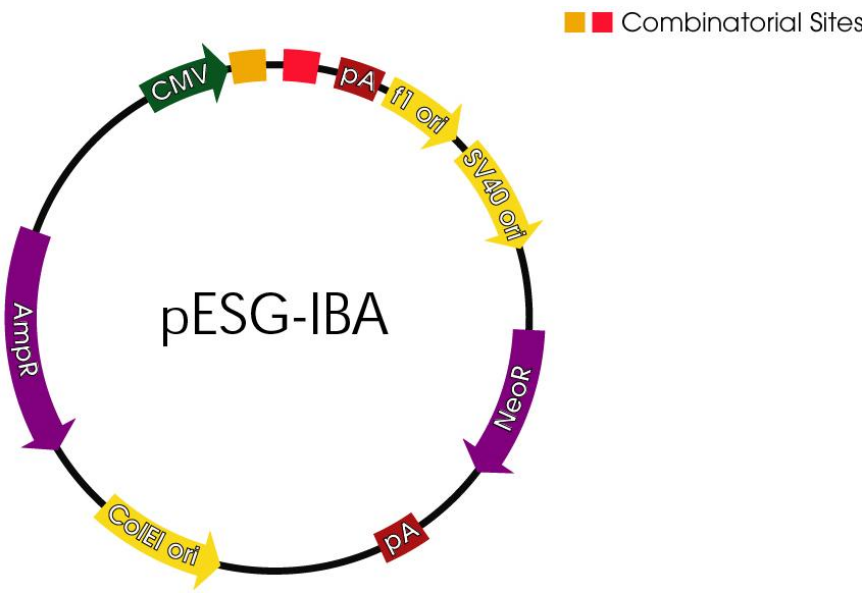
pPSG-IBA vectors which are similar to pPR-IBA vectors use the T7 promoter and T7 RNA polymerase for high-level transcription of the gene of interest (Studier et al., 1990). As the T7 promoter is stronger than the *tet* promoter, pPSG-IBA vectors are recommended in cases where expression with the *tet* 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 pESG-IBA

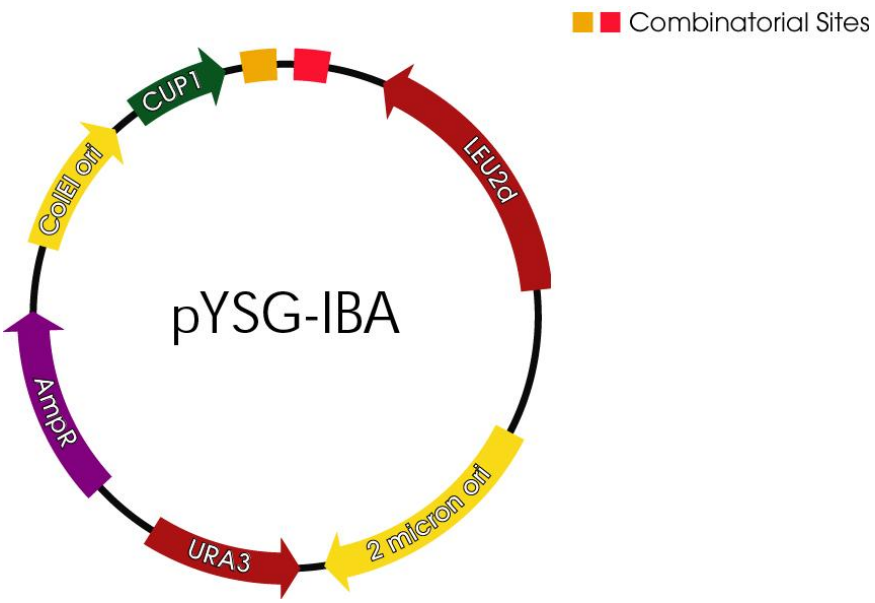


pESG-IBA vectors are designed for high-level constitutive expression of recombinant proteins in a wide range of mammalian host cells through the human cytomegalovirus immediate-early (CMV) promoter (Boshart et al., 1985; Nelson et al., 1987). To prolong expression in transfected cells, the vector will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

5.5 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.

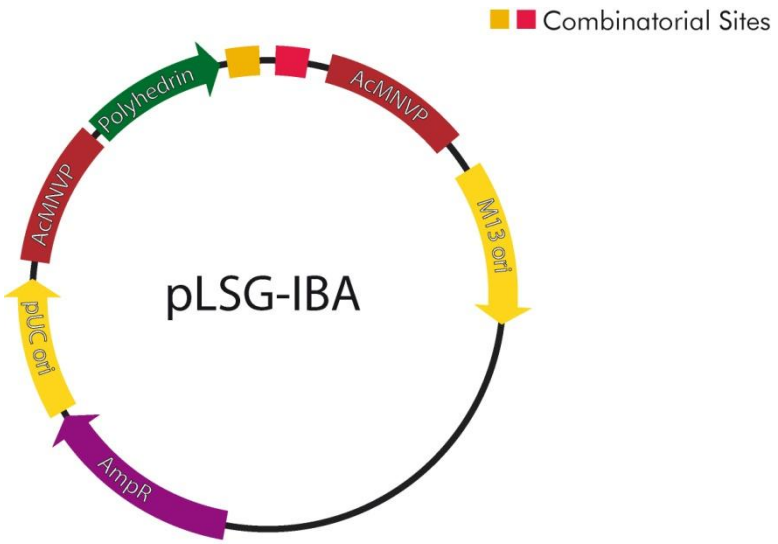
In addition, all vectors include the yeast selectable markers leu2-d (a LEU2 gene with a truncated, but functional promoter) and URA3. Vectors including the leu2-d marker are maintained at high copy number to provide enough gene products from the inefficient promoter for cell survival during growth selection 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

5.6 pLSG-IBA



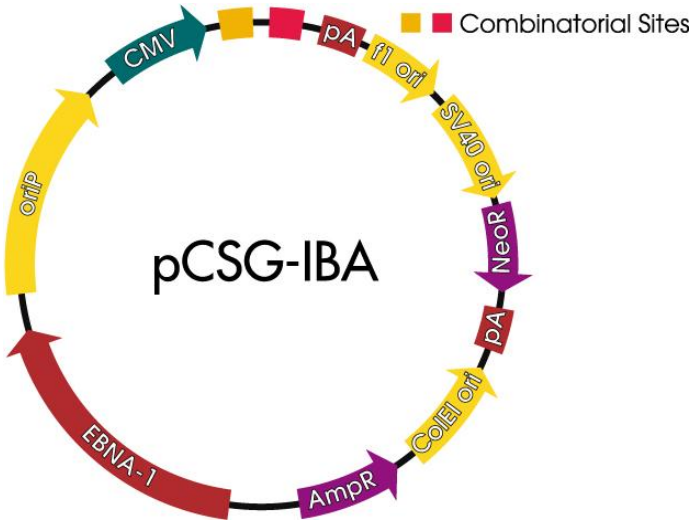
pLSG-IBA vectors are transfer vectors to introduce the GOI into the polyhedrin gene locus of AcMNPV DNA by homologous recombination. Co-transfection with BacPAK6 linearized AcMNPV DNA (Clontech) or circular *flash*BAC modified AcMNPV DNA (Oxford Expression Technologies) allows the generation of recombinant 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

5.7 pCSG-IBA

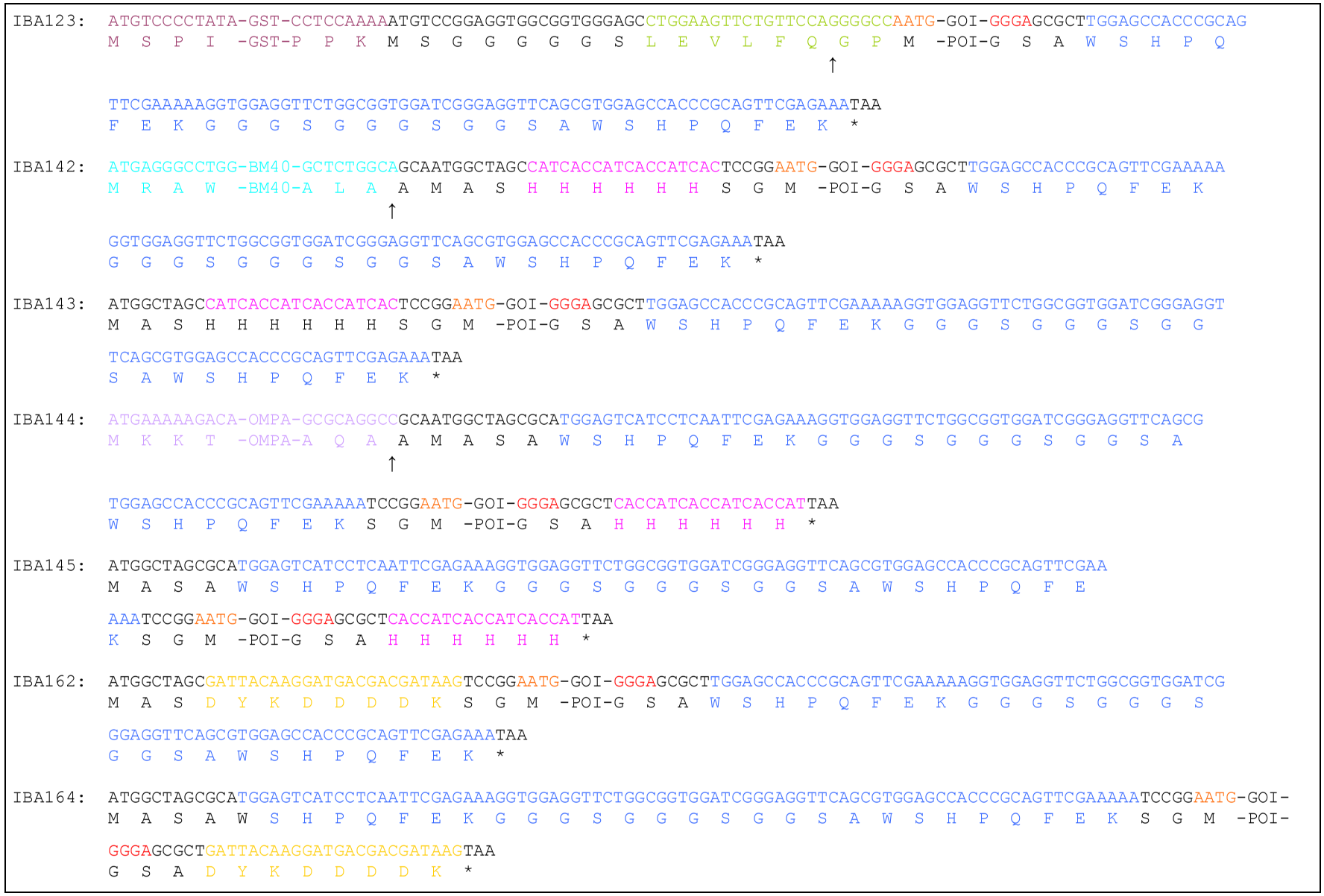


pCSG-IBA vectors are designed for high-level constitutive expression of recombinant proteins in a wide range of mammalian host cells through the human cytomegalovirus immediate-early (CMV) promoter (Boshart et al., 1985; Nelson et al., 1987). The Epstein Barr Virus replication origin (oriP) and nuclear 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

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 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 G G
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 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 S G 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 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 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

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

IRES:
cgaattaattccggttatttccaccatattgccgtctttggcaatgtgagggcccggaaacctggccctgtcttcttgacgagcattcctaggggtctttcccctctgccaaaggaatgcaaggtctgttgaatgtcgtgaaggaagcagttcctctggaagcttcttgaa
gacaaacaacgtctgtagcgaccctttgcaggcagcggaacccccacctggcgacaggtgcctctgcggccaaaagccacgtgtataagatacacctgcaaaggcggcacaaccccagtgccacgttgtgagttggatagttgtggaaagagtcaaatggctcctcc
aagcgtattcaacaagggctgaaggatgcccagaaggtaccccattgtatgggatctgatctgggcctcggtgcacatgctttacatgtgtttagtcgaggttaaaaaacgtctaggccccccgaaccacggggacgtggttttcctttgaaaacacg
```

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

Boshart, M, Weber, F, Jahn, G, Dorsch-Häsler, K, Fleckenstein, B, and Schaffner, W (1985). A Very Strong Enhancer is Located Upstream of an Immediate Early Gene of Human Cytomegalovirus. Cell 41, 521-530.

Butt, TR, Ecker, DJ (1987). Yeast metallothionein and applications in biotechnology. Biotechnol. Reviews 51, 351-364.

Gietz, RD, Sugino, A (1988). New yeast-Escherichia coli shuttle vectors constructed with in vitro mutagenized yeast genes lacking six-base pair restriction sites. Gene 74, 527-34.

Macreadie, IG, Horaitis, O, Verkuylen, AJ, Savin, KW (1991). Improved shuttle vectors for cloning and high-level Cu(2+)-mediated expression of foreign genes in yeast. Gene 104, 107-11.

Nelson, JA, Reynolds-Kohler, C, and Smith, BA (1987). Negative and Positive Regulation by a Short Segment in the 5´-Flanking Region of the Human Cytomegalovirus Major Immediate-Early Gene. Mol. Cell. Biol. *7*, 4125-4129.

Skerra, A. (1994). Use of the tetracycline promoter for the tightly regulated production of a murine antibody fragment in *Escherichia coli*. Gene 151, 131-135.

Studier, FW, Rosenberg, AH, Dunn, JJ, Dubendorff, JW (1990). Use of the T7 RNA polymerase to direct expression of cloned genes. Meth. Enzymol. 185, 60-89.