Protein Production & Assays

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Protocol

Classic cloning with pASK-IBA

General protocol for cloning genes into vectors via the multiple cloning site

1 DESCRIPTION

pASK-IBA vectors are devised for *E. coli* expression of recombinant proteins fused with the Strep-tag®II. They include the chloramphenicol resistance cassette for selection of transformed *E. coli* cells, F1 and ColE1 origin for a high plasmid copy number, a multiple cloning site with recognition sites for several common restriction enzymes (e.g., *EcoR*I, *Sma*I, *Bam*HI, *Sal*I, *Pst*I), and the inducible tetracycline promoter/operator for the regulated expression of proteins. Since the *tet*-promoter works independently of the genetic background of *E. coli*, pASK-IBA vectors can be combined with any *E. coli* strain. Expression of the recombinant protein is induced by addition of anhydrotetracycline, which is a derivative of tetracycline that does not exhibit antibiotic activity. Depending on the specific vector, the protein will be localized in either the cytoplasm or periplasm.

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2 GENERAL INFORMATION

2.1 PCR with *Pfu* polymerase

PCR instructions given in this protocol are recommended for the use of *Pfu* polymerase. When using another Polymerase than *Pfu*, please refer to the recommendations of the respective manufacturer. “Hot-start” DNA polymerase is inactive until the initial denaturation step of PCR cycling. This reduces non-specific priming or the formation of primer dimers. Using 3’-phosphorothioate (PTO) protected oligonucleotides is recommended to protect against the 3’-exonuclease activity of proof-reading polymerases.

Essential parameters for PCR optimization are the annealing temperature, the duration of synthesis and the template concentration. Primers should have a theoretical melting temperature between 60 °C and 70 °C. 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). Additionally, to the priming sequence a non-priming 5’ extension for target vector specific cloning has to be appended to each primer as further specified in Appendix 4.1 for pASK-IBA3C. The annealing temperature should be chosen at least 5 °C below the melting temperature of each primer. The number of cycles should be kept as low as possible to minimize base substitutions.

2.2 Cloning via the multiple cloning site

Foreign genes can be introduced to pASK-IBA vectors after PCR via the multiple cloning site and standard restriction enzymes. The reading frame of the corresponding vector has to be considered if standard restriction enzymes are used. In some vectors with N-terminal Strep-tag®II, the tag is followed by the linker sequence 5'-GGCGCC. This sequence is recognized by three different restriction enzymes generating 5’-overhangs. Cleavage with the suitable enzyme and, if necessary, a subsequent filling reaction makes the production of blunt ends possible in all reading frames.

Using standard restriction sites, additional polylinker derived amino acids are appended at the respective end of the recombinant protein. To avoid the fusion of such polylinker derived amino acids pASK-IBA vectors offer a general cloning strategy via Type IIS restriction enzymes, *Bsa*I or *Eco*31I (NEB, Thermo Scientific™). They allow the precise fusion of the structural gene with the vector encoded functional elements (depending on the vector, Strep-tag®II, OmpA/BM40-signal sequence, protease cleavage site, start codon, or stop codon). To accomplish this, it is necessary to adapt the structural gene at both ends of the coding region via PCR (see cloning scheme at https://www.iba-lifesciences.com/stargate-cloning/#nav-typeiis-restriction-enzymes). To avoid the incorporation of base substitutions, PCR should be performed with a proof-reading DNA polymerase (e.g., *Pfu*) using PTO protected primers.

3 PROTOCOL

3.1 PCR with *Pfu* polymerase

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

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

Concentration	Reagent
200 µM	dNTP
0.1-0.5 µM	Forward primer
0.1-0.5 µM	Reverse primer
5 µM	10x buffer (supplier)
20-200 pg/µl plasmid DNA **or** 0.1-1 ng/µl cDNA library	Template DNA
2.5 U	*Pfu* polymerase*
Ad 50 µl	Distilled H_2O

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

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

3.1.3 Start temperature cycling:

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

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

Alternatively, quantify via photometric measurement.

3.1.5 Isolate the PCR product from the gel. If multiple bands are visible after gel electrophoresis, only isolate the fragment of the expected size. Use of a DNA purification kit to extract the PCR fragment is recommended, since following elution with H2O restriction with Eco31I (BsaI) can be performed immediately.

3.2 Cleavage of PCR fragment

The pASK-IBA vectors can be digested with the isoschizomers BsaI or Eco31I. Since both enzymes show different cutting efficiencies regarding the DNA source (vector DNA or PCR fragment) and incubation time, we determined the cloning efficiency for different digestion protocols. We recommend using BsaI for 1 hour or Eco31I for 16 hours for the cleavage of both the PCR fragment and the vector. For a detailed cloning scheme see Appendix 4.1.

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

Amount	Reagent
X µl	PCR fragment in H_2O (spin eluate)
5 µl	10x *Eco*31I (or *Bsa*I) restriction buffer
10-20 U/µg DNA	Restriction enzyme *(Eco*31I or *Bsa*I)
ad 50 µl	distilled H_2O

3.2.2 Incubate with BsaI at 50 °C for 1 hour (or Eco31I at 37 °C for 16 hours). Take measures to avoid evaporation.

3.2.3 Purify the desired fragment by using a DNA purification kit.

3.2.4 Quantify PCR fragment concentration to determine the appropriate vector to insert-ratio.

3.3 Cleavage of Vector

3.3.1 Mix the following reagents in a 500 µl PCR tube and a total volume of 50 µl:

Amount	Reagent
2 µg	Vector DNA
5 µl	10x *Eco*31I (or *Bsa*I) restriction buffer
10-20 U	Restriction enzyme *(Eco*31I or *Bsa*I)
ad 50 µl	distilled H_2O

3.3.2 Incubate with BsaI at 50 °C for 1 hour (or Eco31I at 37 °C for 16 hours). Take measures to avoid evaporation.

3.3.3 Incubate with PstI for further 30 min at 37 °C or dephosphorylate linearized vector DNA with alkaline phosphatase according to the manufacturer's recommendations. This step is to reduce background colonies which result from re-ligated vector.

3.3.4 Purify vector fragment using a preparative agarose gel and a suitable DNA purification kit.

3.3.5 Quantify vector fragment concentration to determine the appropriate vector to insert-ratio.

3.4 Ligation of PCR fragment and vector

We strongly recommend preparing a negative control without the addition of PCR fragment to quantify background reactions. For ligation use PCR fragment and vector in a molar ratio of 3:1.

3.4.1 Mix the following reagents in a 500 µl reaction tube and a total volume of 20 µl:

Positive control	Negative control	Reagent
100 ng	100 ng	Vector DNA, digested
50 fmol	-	PCR fragment, digested
2 µl	2 µl	Ligation buffer, 10x
1 U	1 U	T4 DNA ligase
ad 20 µl	ad 20 µl	distilled H_2O

3.4.2 Incubate overnight at 16 °C. Heat inactivation is not recommended and not necessary.

3.4.3 Store the sample at 4 °C until transformation.

3.4.4 After initial clone selection (DNA mini preparation/restriction analysis), proceed to DNA sequencing.

3.5 Sequencing

For validating correct vector insertion and sequence of the PCR fragment, the following sequencing primers can be used:

Forward: `5'-GAGTTATTTTACCACTCCCT-3'`
Reverse: `5'-CGCAGTAGCGGTAAACG-3'`

The sequencing primers are also suitable for cycle sequencing.

4 APPENDIX

4.1 Cloning Scheme for the use of *Bsa*I or *Eco31*I

Precise fusion using *Bsa*I for pASK-IBA3C

1. Identification of start and stop codon of the target gene

```
                start codon      target gene       stop codon
                5´-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-TAA-3´
                3´-ATG-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-ATT-5´
```

2. Primer construction

```
Forward primer
    BsaI overhang
 5´-NNNNNN[GGTCTCN]A-ATG-CCCCCCCCCCCCCCCCC
                5´-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-TAA-3´
                3´-ATG-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-ATT-5´
                                GGGGGGGGGGGGGGGGGGG-TCGCGN[CTCTGG]NNNNNN-5´
                                                     BsaI overhang
                                                        Reverse primer
```

3. PCR amplification

```
5´-NNNNNN[GGTCTCN]´A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A´GCGC-N[GAGACC]NNNNNN-3´
3´-NNNNNN[CCAGAGN]-T-TAC´GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-T-CGCG´N[CTCTGG]NNNNNN-5´
           BsaI  BsaI                                        BsaI  BsaI
    recognition  cleavage                               cleavage  recognition
           site  site                                       site  site
```

4. *Bsa*I digested PCR product

```
               5´-A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A-3´
                     3´-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-T-CGCG-5´
                   BsaI                                  BsaI
                   cleaved                               cleaved
                   site                                  site
```

5. Ligation with *Bsa*I digested pASK-IBA3C

```
5´-AACGAGGGCAAAA`                                    ´gcgc-T-TGGAGCCACCCGCAGTTCGAAAAA-TAA-3´
3´-TTGCTCCCGTTTT-T-TAC`                                   ´A-ACCTCGGTGGGCGTCAAGCTTTTT-ATT-5´
       ribosome    Met                                Ser-Ala         Strep-tag®II     STOP
       binding                                        linker         8 amino acids
       site                                      2 amino acids
```

6. Ligated construct

```
5´-AACGAGGGCAAAA-A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A-gcgc-T-TGGAGCCACCCGCAGTTCGAAAAA-TAA-3´
3´-TTGCTCCCGTTTT-T-TAC-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-T-CGCG-A-ACCTCGGTGGGCGTCAAGCTTTTT-ATT-5´
      ribosome     Met           target gene           Ser-Ala         Strep-tag®II     STOP
      binding                                          linker         8 amino acids
      site                                        2 amino acids
```

4.2 Multiple Cloning sites of pASK-IBA vectors

pASK-IBA2C

```
1      CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCA   80
                                                                  forward primer

                                                                           OmpA
                                                                  M  K  K  T  A  I  A
81     GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAAATGAAAAAGACAGCTATCGCGA   160
                                             XbaI

                    OmpA
       I  A  V  A  L  A  G  F  A  T  V  A  Q  A  G  D  H  G  P  E  F  E  L  G  T  R  G
161    TTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAggccGGAGACCATGGTCCCGAATTCGAGCTCGGTACCCGGGGA   240
                                                  BsaI      BsmFI     SstI  KpnI     BamHI
                                                    PshAI       EcoRI            SmaI
                                                      NcoI

                                             link        Strep-tag®II
        S  L  E  V  D  L  Q  G  D  H  G  L  S  A  W  S  H  P  Q  F  E  K  *
241    TCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTTGGAGCCACCCGCAGTTCGAAAAATAATAAGCTTGACC   320
          XhoI  SalI  PstI   BsmFI   BsaI  Eco47III                          HindIII
                                PshAI
                                  NcoI

321    TGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGCTACTGCGTCACGGATCTCCACGC   400
                                                       reverse primer
```

pASK-IBA3C

```
1      CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCA   80
                                                                  forward primer

                                                                  M  G  D  R  G  P  E
81     GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAaatgGGAGACCGCGGTCCCGAAT   160
                                             XbaI                    BsaI      BsmFI
                                                                       PshAI       EcoRI
                                                                         SacII

                                                                link      Strep-tag®II
       F  E  L  G  T  R  G  S  L  E  V  D  L  Q  G  D  H  G  L  S  A  W  S  H  P  Q  F
161    TCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCAgcgcTTGGAGCCACCCGCAGTTC   240
         SstI  KpnI     BamHI       SalI  PstI   BsmFI   BsaI  Eco47III
                   SmaI       XhoI                 PshAI
                                                     NcoI

        E  K  *
241    GAAAAATAATAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGCTAC   320
                 HindIII                                                    reverse primer

321    TGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTAC   400
```

pASK-IBA4C

```
1       CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCA    80
                                                                   forward primer

                                                                   M  K  K  T  A  I  A
81      GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAAATGAAAAAGACAGCTATCGCGA   160
                                             XbaI

                     OmpA                         link        Strep-tag®II      link   R
        I  A  V  A  L  A  G  F  A  T  V  A  Q  A  A  S  W  S  H  P  Q  F  E  K  G  A  E
161     TTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCCGCTAGCTGGAGCCACCCGCAGTTCGAAAAAGgcgcCGAG   240
                                                 NheI                          BbeI  BsaI
                                                                               EheI    PshAI
                                                                               KasI
                                                                               NarI

        D  R  G  P  E  F  E  L  G  T  R  G  S  L  E  V  D  L  Q  G  D  H  G  L  *
          P  R  S  R  I  R  A  R  Y  P  G  I  P  R  G  R  P  A  G  G  P  W  S  L  I  S
         T  A  V  P  N  S  S  S  V  P  G  D  P  S  R  S  T  C  R  G  T  M  V  S  D  I  *
241     ACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTCTgataTCTA   320
         SacII     EcoRI       KpnI     BamHI       SalI  PstI   BsmFI   BsaI    EcoRV
               BsmFI     SstI      SmaI       XhoI                 PshAI
                                                                     NcoI
        N  *

321     ACTAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGCTACTGCGTCA   400
           HindIII                                                   reverse primer
```

pASK-IBA5C

```
1       CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCA    80
                                                                   forward primer

                                                                      link    Strep-tag®II
                                                                   M  A  S  W  S  H  P
81      GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAAATGGCTAGCTGGAGCCACCCGC   160
                                             XbaI                    NheI

                            D  R  G  P  E  F  E  L  G  T  R  G  S  L  E  V  D  L  Q  G
                    link   R  P  R  S  R  I  R  A  R  Y  P  G  I  P  R  G  R  P  A  G  G
        Q  F  E  K  G  A  E  T  A  V  P  N  S  S  S  V  P  G  D  P  S  R  S  T  C  R  G
161     AGTTCGAAAAAGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGG   240
                   BbeI  BsaI      BsmFI     SstI  KpnI     BamHI       SalI  PstI   BsmFI
                   EheI    PshAI       EcoRI           SmaI       XhoI                 PshAI
                   KasI      SacII
                   NarI

        D  H  G  L  *
          P  W  S  L  I  S  N  *
         T  M  V  S  D  I  *
241     ACCATGGTCTCTgataTCTAACTAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGC   320
         NcoI       EcoRV      HindIII
             BsaI

321     CGTTTACCGCTACTGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCA   400
          reverse primer
```

pASK-IBA6C

```
1          CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTAT   78
                                                                        forward primer

                                                                         M  K  K  T  A  I  A
79        CAGTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAAATGAAAAAGACAGCTATCGC   158
                                                XbaI

                  OmpA                       link       Strep-tag®II       Factor Xa
            I  A  V  A  L  A  G  F  A  T  V  A  Q  A  A  S  W  S  H  P  Q  F  E  K  I  E
159       GATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCCGCTAGCTGGAGCCACCCGCAGTTCGAAAAAATCGAAG   238
                                                    NheI

                  R  P  R  S  R  I  R  A  R  Y  P  G  I  P  R  G  R  P  A  G  G  P  W  S
                 E  T  A  V  P  N  S  S  S  V  P  G  D  P  S  R  S  T  C  R  G  T  M  V  S
          G  R  R  D  R  G  P  E  F  E  L  G  T  R  G  S  L  E  V  D  L  Q  G  D  H  G  L
239       GgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTGCAGGGGGACCATGGTCTC   318
          BbeI  BsaI     BsmFI    SstI  KpnI    BamHI       SalI  PstI   BsmFI   BsaI
          EheI     PshAI      EcoRI           SmaI       XhoI                 PshAI
          KasI       SacII                                                      NcoI
          NarI

          L  I  S  N  *
            D  I  *
           *
319       TgataTCTAACTAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGCT   398
          EcoRV       HindIII                                                 reverse primer

399       ACTGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCT   478
```

pASK-IBA7C

```
1         CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCA   80
                                                                      forward primer

                                                                          link   Strep-tag®II
                                                                        M  A  S  W  S  H  P
81        GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAAATGGCTAGCTGGAGCCACCCGC   160
                                                     XbaI                    NheI

                                    R  P  R  S  R  I  R  A  R  Y  P  G  I  P  R  G  R  P
                 factor Xa         E  T  A  V  P  N  S  S  S  V  P  G  D  P  S  R  S  T  C
          Q  F  E  K  I  E  G  R  R  D  R  G  P  E  F  E  L  G  T  R  G  S  L  E  V  D  L
161       AGTTCGAAAAAATCGAAGGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTG   240
                            BbeI  BsaI     BsmFI    SstI  KpnI    BamHI       SalI  PstI
                            EheI     PshAI      EcoRI           SmaI       XhoI
                            KasI       SacII
                            NarI

          A  G  G  P  W  S  L  I  S  N  *
            R  G  T  M  V  S  D  I  *
           Q  G  D  H  G  L  *
241       CAGGGGGACCATGGTCTCTgataTCTAACTAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTT   320
              BsmFI   BsaI  EcoRV      HindIII
                PshAI
                  NcoI

321       TGTCTGCCGTTTACCGCTACTGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTT   400
                  reverse primer
```

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