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Classic cloning with pASK-IBA

General protocol

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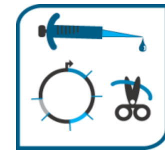
Content

1 Introduction – Cloning of an arbitrary gene into pASK-IBA expression vectors 4
2 PCR with *Pfu* DNA polymerase 5
3 Cloning of a PCR product via *Bsa*I or *Eco*31I (Type IIS restriction enzymes) 7
3.1 PCR product purification 7
3.2 Restriction digest of PCR fragment and Vector using *Bsa*I or *Eco*31I 7
3.3 Ligation reaction 9
3.4 Sequencing 9
4 APPENDIX 10
4.1 Cloning Scheme for the use of *Bsa*I or *Eco31*I 10
4.2 Multiple Cloning Sites of pASK-IBA vectors 11
5 References 13

1 Introduction – Cloning of an arbitrary gene into pASK-IBA expression vectors

The multiple cloning sites of pASK-IBA vectors include many standard unique restriction sites like *Eco*RI or *Bam*HI for the introduction of foreign genes after PCR. However, the reading frame of the corresponding vector has to be considered if such restriction sites are used. In some vectors with N-terminal Strep-tag®II, Strep-tag®II 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, MBI Fermentas). They allow the precise fusion of the structural gene with the vector encoded functional elements (depending on the vector, Strep-tag®II, *Omp*A/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 www.iba-lifesciences.com). In order to avoid the incorporation of base substitutions, PCR should be performed with a proof-reading DNA polymerase (e.g. *Pfu*) using phosphorothioate protected primers.

2 PCR with *Pfu* DNA polymerase



Important notes

- **Annealing and melting temperature of Primers:**
  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 paragraph 4.1 for pASK-IBA3C.
  The annealing temperature should be chosen at least 5°C below the melting temperature of each primer.

- **Cycles:**
  The number of cycles should be kept as low as possible in order to minimize base substitutions.

- **Polymerases:**
  - 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.
  - PTO protected primers: The use of 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.

Protocol

1. **Mix the following reagents in a 500 µl reaction tube and 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.**

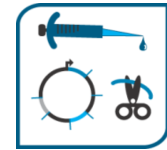
4. **Start temperature cycling:**

94 °C	30 s	Denaturation	Use 15-20 cycles for plasmid DNA and 30-40 cycles for cDNA library
55-65 °C	30-60 s	Annealing	
72 °C	30-240 s	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).**

3 Cloning of a PCR product via *Bsa*I or *Eco*31I (Type IIS restriction enzymes)



3.1 PCR product purification

Protocol	
	1. **Purify PCR product to remove 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 agarose gel electrophoresis. 2. **If a spin kit is used and the PCR fragment is eluted in H_2O, *Eco*31I (*Bsa*I) restriction can be performed immediately.** 1. **Quantify PCR fragment by analytical agarose gel electrophoresis through band intensity comparison with a DNA standard.** Applying two different amounts of PCR product in separate lanes is recommended to find a band of equal intensity with a band of the DNA standard which has to be applied on the same gel for exact quantification.

3.2 Restriction digest of PCR fragment and Vector using *Bsa*I or *Eco*31I

Important notes	
	The pASK-IBA vectors can be digested with the isoschizomers *Bsa*I or *Eco*31I. 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. According to our results, we recommend using *Bsa*I for 1 hour or *Eco*31I for 16 hours for the cleavage of both the PCR fragment and the vector. • For a detailed cloning scheme see Appendix 4.1.

Protocols

Cleavage of PCR-Fragment:

1. **Mix the following reagents in a 500 µl reaction tube and a total volume of 50 µl:**

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

1. **Incubate with *Bsa*I at 50 °C for 1 hour (or *Eco*31I at 37 °C for 16 hours). Take measures to avoid evaporation.**

2. **Purify the desired fragment by using a spin column.**

3. **Quantify PCR fragment concentration to determine the appropriate vector to insert-ratio. Compare the band intensity of both the PCR fragment and the corresponding vector with a DNA standard on the same analytical agarose gel.**

Cleavage of Vector:

1. **Mix the following reagents in a 500 µl reaction tube and total volume of 50 µl:**

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

2. **Incubate with *Bsa*I at 50 °C for 1 hour (or *Eco*31I at 37 °C for 16 hours). Take measures to avoid evaporation.**

3. **Incubate with *Pst*I 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.

4. **Purify vector fragment** using a preparative agarose gel with subsequent spin column purification

5. **Quantify vector fragment concentration to determine the appropriate vector to insert-ratio. Compare the band intensity of both the PCR fragment and the corresponding vector with a DNA standard on the same analytical agarose gel.**

3.3 Ligation reaction

Important notes	
	• To quantify background reactions we strongly recommend preparing a negative control without the addition of PCR fragment. • For ligation use PCR fragment and vector in a molar ratio of 3:1

Protocol

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

Pos. control	Neg. control	
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

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

3. **Store the sample at 4 °C until transformation.**

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

3.4 Sequencing

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

Sequencing primers for pASK-IBA vectors:
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´-NNNNNNGGTCTCNA-ATG-CCCCCCCCCCCCCCCCC
                5´-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-TAA-3´
                3´-ATG-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-ATT-5´
                                  GGGGGGGGGGGGGGGGGGGG-TCGCGNCTCTGGNNNNNN-5´
                                                          BsaI overhang

                                                             Reverse primer
```

3. PCR amplification

```
5´-NNNNNNGGTCTCN´A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A´GCGC-NGAGACCNNNNNN-3´
3´-NNNNNNCCAGAGN-T-TAC´GGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-T-CGCG´NCTCTGGNNNNNN-5´
           BsaI  BsaI                                   BsaI  BsaI
    recognition  cleavage                           cleavage  recognition
           site  site                                   site  site
```

4. Digestion of PCR product with amplification *Bsa*I

```
              5´-A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A-3´
                    3´-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-T-CGCG-5´
           BsaI  BsaI                                   BsaI  BsaI
```

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-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A-gcgc-T-TGGAGCCACCCGCAGTTCGAAAAA-TAA-3´
3´-TTGCTCCCGTTTT-T-TAC-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-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

```
                                                                      forward primer
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
```

5 References

For help with DNA ligation, *E. coli* transformation, restriction enzyme analysis, purification of DNA, DNA sequencing, and DNA biochemistry, please refer to “Molecular Cloning: A Laboratory Manual” (Sambrook et al., 1989) or “Current Protocols in Molecular Biology” (Ausubel et al., 1994).

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