

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., *EcoRI*, *SmaI*, *BamHI*, *SalI*, *PstI*), 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.

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/applications/cloning-expression/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	<i>Pfu</i> polymerase*
Ad 50 µl	Distilled H ₂ O

*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 *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. 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.

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 ₂ O (spin eluate)
5 µl	10x <i>Eco</i> 31I (or <i>Bsa</i> I) restriction buffer
10-20 U/µg DNA	Restriction enzyme (<i>Eco</i> 31I or <i>Bsa</i> I)
ad 50 µl	distilled H ₂ O

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

3.2.3. 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 <i>Eco</i> 31I (or <i>Bsa</i> I) restriction buffer
10-20 U	Restriction enzyme (<i>Eco</i> 31I or <i>Bsa</i> I)
ad 50 µl	distilled H ₂ O

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

3.3.3. 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.

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 ₂ O

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. Troubleshooting

4.1. Cloning Scheme for the use of *BsaI* or *Eco31I*

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

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start codon      target gene      stop codon
5'-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-TAA-3'
3'-ATG-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-ATT-5'
  
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4.1.2. Primer construction

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Forward primer
BsaI overhang
5'-NNNNNNGGTCTCNA-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-TAA-3'
                    5'-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-TAA-3'
                    3'-ATG-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-ATT-5'
                                GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-TCGCGNCTCTGGNNNNNN-5'
                                                BsaI overhang
                                                Reverse primer
  
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4.1.3. PCR amplification

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5'-NNNNNNGGTCTCN'A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A'GCGC-NGAGACCNNNNNN-3'
3'-NNNNNNCCAGAGN-T-TAC'GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-T-CGCG'NCTCTGGNNNNNN-5'
      BsaI   BsaI                                BsaI   BsaI
      recognition cleavage                        cleavage recognition
      site      site                                site      site
  
```

4.1.4. *BsaI* digested PCR product

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5'-A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A-3'
3'-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-T-CGCG-5'
      BsaI                                BsaI
      cleaved                          cleaved
      site                              site
  
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4.1.5. Ligation with *BsaI* digested pASK-IBA3C

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5'-AACGAGGGCAAAA`                                `gcgc-T-TGGAGCCACCCGAGTTCGAAAAA-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
  
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4.1.6. Ligated construct

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5'-AACGAGGGCAAAA-A-ATG-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-A-gcgc-T-TGGAGCCACCCGAGTTCGAAAAA-TAA-3'
3'-TTGCTCCCGTTTT-T-TAC-GGGGGGGGGGGGGGGGGGGGGGGGGGGGGGGG-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	CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCCTCCCTATCA	80
	forward primer	
	OmpA	
	M K K T A I A	
81	GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAATGAAAAGACAGCTATCGCGA	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	TCCCTCGAGGTGCACCTGCAGGGGGACCATGGTCTCAGcgCTTGGAGCCACCCGAGTTCGAAAAATAATAAGCTTGACC	320
	XhoI SalI PstI BsmFI BsaI Eco47III HindIII	
	PshAI	
	NcoI	
321	TGTGAAGTGAAAATGGCGCACATTGTGCGACATTTTTTTGTCTGCCGTTTACCGCTACTGCGTCACGGATCTCCACGC	400
	reverse primer	

pASK-IBA3C

1	CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCCTCCCTATCA	80
	forward primer	
	M G D R G P E	
81	GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAatGGAGACCGGTCCCGAAT	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	TCGAGCTCGGTACCCGGGGATCCCTCGAGGTGCACCTGCAGGGGGACCATGGTCTCAGcgCTTGGAGCCACCCGAGTTC	240
	SstI KpnI BamHI SalI PstI BsmFI BsaI Eco47III	
	SmaI XhoI PshAI	
	NcoI	
	E K *	
241	GAAAAATAATAAGCTTGACCTGTGAAGTGAAAATGGCGCACATTGTGCGACATTTTTTTGTCTGCCGTTTACCGCTAC	320
	HindIII reverse primer	
321	TGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAAGCGGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTAC	400

pASK-IBA4C

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1      CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTTGACACTCTATCATTGATAGAGTTATTTTACCACTCCCTATCA      80
                                     forward primer

                                     M K K T A I A
81      GTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAAATGAAAAGACAGCTATCGCGA      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      TTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCCGCTAGCTGGAGCCACCCGAGTTCGAAAAAGgcgcCGAG      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 G T M V S D I *
241      ACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTGCACCTGCAGGGGACCATGGTCTCTgataCTA      320
      SacII EcoRI KpnI BamHI SalI PstI BsmFI BsaI EcoRV
      BsmFI SstI SmaI XhoI PshAI
      NcoI

N *
321      ACTAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTTGTCTGCCGTTTACCGCTACTGCGTCA      400
      HindIII reverse primer
  
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pASK-IBA5C

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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      AGTTCGAAAAAGgcgcCGAGACCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTGCACCTGCAGGGGG      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

CGTTTACCGCTACTGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCA      400
reverse primer
  
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pASK-IBA6C

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1      CCATCGAATGGCCAGATGATTAATTCCTAATTTTGTGACTCTATCATTGATAGAGTTATTTTACCCTCCCTAT 78
                                     forward primer

                                     M K K T A I A
79      CAGTGATAGAGAAAAGTGAAATGAATAGTTCGACAAAAATCTAGATAACGAGGGCAAAAAATGAAAAGACAGCTATCGC 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     GATTGCAGTGGCACTGGCTGGTTTCGCTACCGTAGCGCAGGCCGCTAGCTGGAGCCACCCGCAGTTCGAAAAATCGAAG 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     TgataTCTAACTAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTTGTCTGCCGTTTACCGCT 398
EcoRV      HindIII          reverse primer

399     ACTGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCT 478

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pASK-IBA7C

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1      CCATCGAATGGCCAGATGATTAATTCCTAATTTTTGTGACTCTATCATTGATAGAGTTATTTTACCCTCCCTATCA 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     AGTTCGAAAAATCGAAGGgcgcCGAGACCGCGGTCCCGAATTCGAGCTCGGTACCCGGGGATCCCTCGAGGTCGACCTG 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     CAGGGGACCATGGTCTCTgataTCTAACTAAGCTTGACCTGTGAAGTGAAAAATGGCGCACATTGTGCGACATTTTTTT 320
                                     BsmFI  BsaI  EcoRV      HindIII
                                     PshAI
                                     NcoI

321     TGTCTGCCGTTTACCGCTACTGCGTCACGGATCTCCACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTT 400
                                     reverse primer

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