

# PCR cloning with pASK-IBA, pPR-IBA and pEXPR-IBA vectors

Last date of revision  
May 2009

Version PR08-0007

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## General protocol for PCR cloning with pASK-IBA, pPR-IBA and pEXPR-IBA vectors

### General remarks – Cloning of an arbitrary gene into pASK-IBA expression vectors

The multiple cloning sites of pASK-IBA, pPR-IBA and pEXPR-IBA vectors include many standard unique restriction sites like *EcoRI* or *BamHI* 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 3 different restriction enzymes generating 5'-overhangs. Cleavage with the suitable enzyme and, if necessary, a subsequent filling reaction makes the production of blunt ends in all reading frames possible. 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, pPR-IBA and pEXPR-IBA vectors offer a general cloning strategy via Type IIS restriction enzymes, *BsaI* or *Eco31I* (NEB, MBI Fermentas). They allow the precise fusion of the structural gene with the vector encoded functional elements (*Strep-tag II* and, depending on the vector, *OmpA*-signal sequence, protease cleavage site, His-tag, 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 (s. cloning scheme in the IBA catalogue or at [www.iba-go.com](http://www.iba-go.com)). In order to avoid the incorporation of base substitutions, PCR should be performed with a proof reading DNA polymerase (e.g. *Pfu*, Stratagene) using phosphorothioate protected primers. The essential primer sequences to introduce the *BsaI* restriction site into the PCR fragment for the cloning with a certain vector can be easily determined with our "Primer Design Software" which is free of charge and can be downloaded at IBA's web site.

## PCR with *Pfu* DNA polymerase

Standard PCR assay; hot-start; PTO protected primers

Mix the following reagents in a 500  $\mu$ l reaction tube:

		final concentration:	
dNTP (10 mM each)	1 $\mu$ l	200 $\mu$ M	
Forward primer (10 $\mu$ M)	2.5 $\mu$ l	500 nM	
Reverse primer (10 $\mu$ M)	2.5 $\mu$ l	500 nM	
10x buffer (supplier)	5 $\mu$ l		
Template DNA	X $\mu$ l	20 to 200 $\mu$ g/ $\mu$ l	(plasmid DNA)
		0,1 to 1 ng/ $\mu$ l	(cDNA library)
H <sub>2</sub> O	to 50 $\mu$ l		

Overlay the sample with 50  $\mu$ l mineral oil

Heat the sample at 94 °C for 3 min

Add 1  $\mu$ l *Pfu* DNA polymerase (2.5  $\mu$ l/ $\mu$ l)

Start temperature cycling.

Anneal and denature for 30 sec or 1 min. Since the rate of synthesis of *Pfu* is significantly slower than that of *Taq*, the duration of the DNA synthesis step should be doubled when using *Pfu* in comparison to protocols utilizing *Taq* polymerase (further information can be obtained from the manufacturer Stratagene). The annealing temperature depends on the primer melting temperatures which 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). Primers should have a theoretical melting temperature between 60 °C and 70 °C (this will be achieved automatically if the "Primer Design Software" is used). PCR annealing should be performed at 55 °C.

If plasmid DNA with already cloned gene is used as a template, 15 to 20 cycles are usually sufficient, while it is recommended to use between 30 and 40 cycles when cDNA libraries are used as a template. Generally, the number of cycles should be kept as low as possible in order to minimize the possibility of the incorporation of base substitutions. A final 60°C incubation should be performed for 5 min in order to obtain full length products. Samples are stored at 4°C until agarose gel electrophoresis.

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

## Cloning of a PCR product via Type IIS restriction enzymes, *Bsal* or *Eco311*

First, the PCR product should be purified. The purification step is recommended to create optimal buffer conditions for effective cleavage of the PCR product. If PCR produced a single product, purification can be performed using a spin kit (e.g. Biometra order-no. 4100-460B) without prior separation on an agarose gel. Otherwise, a preparative agarose gel is essential for purification. If a spin kit is used and the DNA fragment is eluted in H<sub>2</sub>O, *Bsal* restriction can be performed immediately without any precipitation step.

pASK-IBA, pPR-IBA and pEXPR-IBA vectors can be digested with the isoschizomers *Bsal* or *Eco311*. However, both enzymes show different cutting efficiencies regarding the DNA source (vector DNA or PCR fragment) and the incubation time. Therefore we performed a comparison of *Bsal* vs. *Eco311* and determined the cloning efficiency by counting the resulting colonies after transformation of the ligation reaction into DH5alpha cells. As a result, we recommend to use *Bsal* for 1 hour or *Eco311* for 16 hours for the cleavage of both the PCR fragment and the vector.

			pASK-IBA3			
			<i>Bsal</i>		<i>Eco311</i>	
			1 h	16 h	1 h	16 h
PCR fragment	<i>Bsal</i>	1 h	<b>1208</b>	<b>1028</b>	<b>265</b>	<b>291</b>
		16 h	<b>92</b>	<b>51</b>	<b>22</b>	<b>10</b>
	<i>Eco311</i>	1 h	<b>77</b>	<b>2</b>	<b>12</b>	<b>8</b>
		16 h	<b>1271</b>	<b>1228</b>	<b>952</b>	<b>1140</b>
no PCR fragment (control)			<b>0</b>	<b>0</b>	<b>0</b>	<b>0</b>

Table 1: Determining the cloning efficiency of a PCR fragment into pASK-IBA3 using *Bsal* or *EcoRI*. (Counted colonies are indicated in bold.) The vector pASK-IBA3 has been digested by *Bsal* and *Eco311* for 1 or 16 hours, respectively (see columns). To reduce background the linearized vector was dephosphorylated using shrimp alkaline phosphatase. The DNA has been purified via an agarose gel and was ligated to PCR fragments which have been digested in the same way (see rows). After overnight incubation at 16°C the ligation reaction was transformed into DH5alpha cells and plated onto LB/ampicillin plates. The resulting colonies were counted.

## Restriction digest protocols for cloning using *Bsal* or *Eco311*

### 1) PCR fragment

For restriction digest of the **PCR fragment** add 5  $\mu$ l 10x *Eco311* (or *Bsal*) restriction buffer to the spin eluate, respectively.

Add H<sub>2</sub>O and restriction enzyme ad 50  $\mu$ l, at a rate of 10 to 20 units per  $\mu$ g DNA. Overlay with mineral oil and incubate at 37 °C with *Eco311* for 16 h (or at 50 °C with *Bsal* for 1 h), respectively. After restriction, the desired fragment is purified using a spin column. 10 % of the eluate is applied onto analytical agarose gel together with a DNA standard for quantification.

### 2) Vector

For restriction digest of the **vector** incubate 2  $\mu$ g vector DNA with 10 to 20 units *Bsal* at 50 °C for 1 hour (or *Eco311* at 37 °C for 16 hours).

To reduce number of background colonies which result from re-ligated vector either incubate with *Pst*I for further 30 min at 37 °C or dephosphorylate linerized vector DNA with phosphatase (e.g. shrimp alkaline phosphatase from USB) according to the manufacturers recommendations.

After restriction, the desired vector fragment is purified using a preparative agarose gel with subsequent spin purification whereas the PCR fragment may be purified using a spin column without prior agarose gel separation. 10 % of the eluates are applied onto analytical agarose gel together with a DNA standard for quantification.

## Ligation reaction

To quantify the background reactions we strongly recommend to perform a negative control without the addition of PCR fragment.

	Pos. control	Neg. control
Vector DNA, digested	100 ng	100 ng
PCR fragment, digested	50 fmol	-
Ligation buffer, 10x	2 $\mu$ l	2 $\mu$ l
T4 DNA ligase	1 unit	1 unit
H <sub>2</sub> O	to 20 $\mu$ l	to 20 $\mu$ l

Incubate overnight at 16 °C and store the sample at 4 °C until transformation. Heat inactivation is not recommended and not necessary.

After transformation and screening for a correct clone by DNA mini preparation and subsequent restriction analysis, proceed to DNA sequencing.

Sequencing primers for pASK-IBA vectors (cat. No. 5-0000-103):

Forward: 5' -GAGTTATTTTACCACTCCCT-3'

Reverse: 5' -CGCAGTAGCGGTAAACG-3'

Sequencing primers for pPR-IBA vectors (cat. No. 5-0000-113):

Forward: 5' -TAATACGACTCACTATAGGG-3'

Reverse: 5' -TAGTTATTGCTCAGCGGTGG-3'

Sequencing primers for pEXPR-IBA vectors (cat. No. 5-0000-123):

Forward: 5' -GAGAACCCACTGCTTACTGGC-3'

Reverse: 5' -TAGAAGGCACAGTCGAGG-3'

The sequencing primers are also suitable for cycle sequencing.

For help with DNA ligations, *E. coli* transformations, 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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