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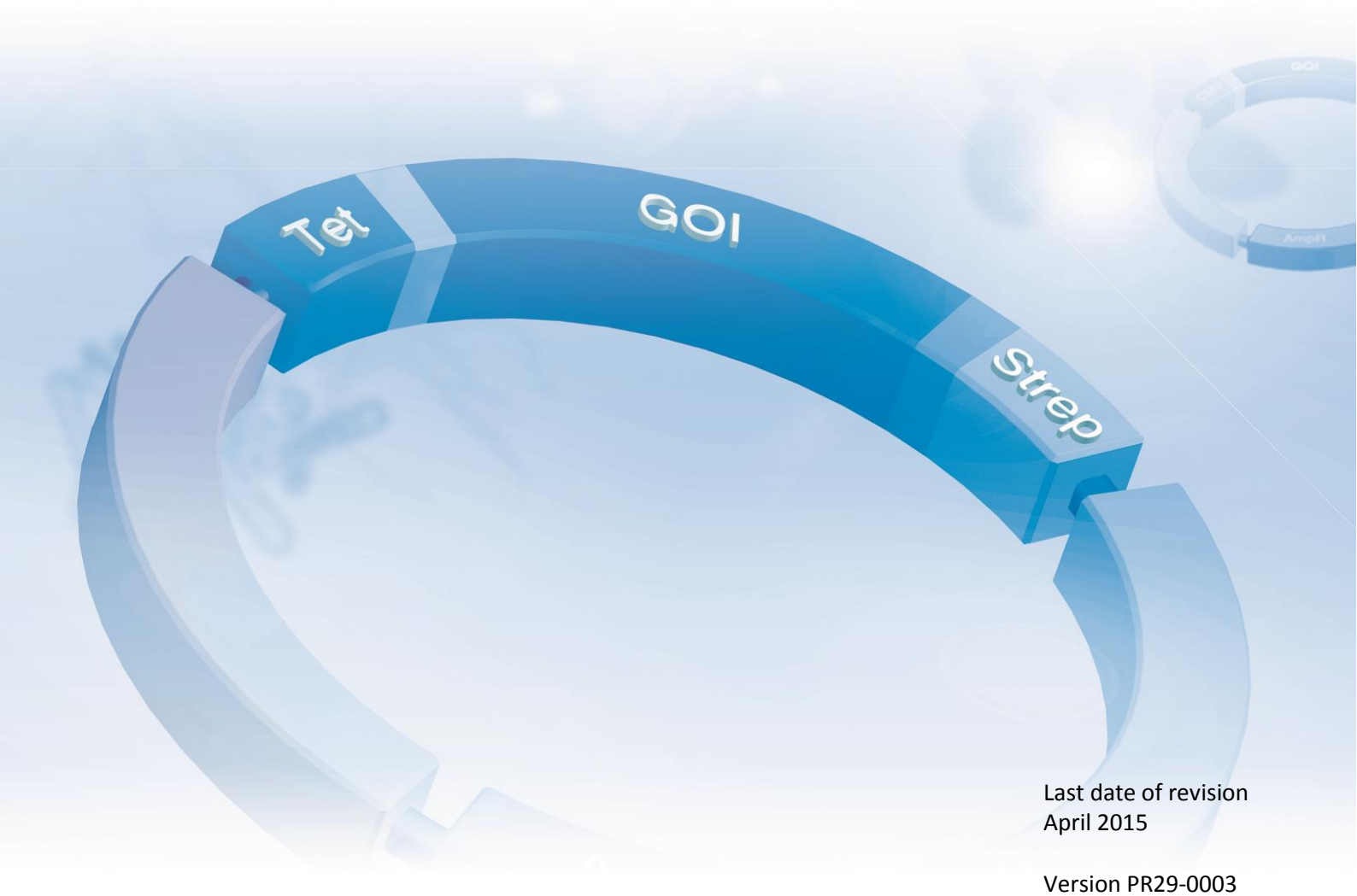
For Life Sciences

Generation of gene knockout vectors for *Dictyostelium discoideum*

Instruction manual



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1 Introduction

Primer design for the generation of PCR products from genomic DNA of *D. discoideum*

The left arms (LA) and right arms (RA) for homologous recombination have to be equipped in a first step by PCR at both termini with combinatorial sites and the Esp3I recognition site, which are important for oriented insertion of the PCR fragment into pKOSG-IBA-Dicty1

The required primers consist of the Esp3I recognition site (light blue underlined), either of the combinatorial sites (CS1-4), a unique restriction enzyme recognition site (RS, as indicated in Table 1) and the sequence to be amplified. The sequence length that is complementary to the fragment determines the melting temperature. A melting temperature between 50°C and 55°C should be achieved, using the following rule of thumb that each A:T-pair contributes 2°C and each G:C-pair 4°C.

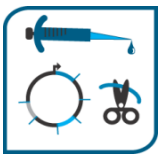
Table 1: General primer design for pKOSG-IBA-Dicty

primer name	combinatorial site	SEQUENCE OF PRIMER (5'→3')A
LA1	CS1	AGCGCGTCTCCAATG - **unique RS -** forward sequence LA
LA2	CS2	AGCGCGTCTCCGTTG - reverse sequence LA
RA1	CS3	AGCGCGTCTCCCTTC - forward sequence RA
RA2	CS4	AGCGCGTCTCCTCCC - **unique RS -** reverse sequence RA

Note: RS denotes the unique restriction enzyme recognition sequence site that is added to the primer for plasmid analysis and cloning in *Dictyostelium discoideum*. It must not occur in either the left or the right arm.

Whenever possible, the recognition sequence (RS) of the same restriction enzyme should be added to the LA1 and RA2 primers. In the context of the pKOSG-IBA-Dicty1, suitable restriction enzymes are for example *Bgl* I, *Bpu* 1102 I, *Eco* RI, *Kpn* I, *Pst* I. Among these any can be chosen whose recognition sequence does not occur in either of the selected arms for homologous recombination.

2 Protocol



Required products	Cat.No.	Amount
Competent *E. coli* TOP 10 cells	5-1600-020	20 reactions
pKOSG-IBA-Dicty1	5-1650-005	5 reactions

Products from other suppliers
ATP
DTT
T4 DNA Ligase [1 U/µl]
*Esp*3I [10 U/µl] restriction enzyme and supplied buffer (e.g. Thermo Scientific; The enzyme must be active at 37°C. Do not use the isoschizomer *Bsm*BI.)

Important notes

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

Protocol

PCR reaction

1. **Mix the following reagents in a 500 µl reaction tube and total volume of 50 µl** (based on standard protocols for *Pfu* Polymerase PCR):

200 µM	dNTP (each)
500 nM	Forward primer
500 nM	Reverse primer
5 µl	10x buffer (supplier)
10 - 100 ng/µl	Template DNA (genomic DNA)
2.5 U	*Pfu* DNA polymerase (depending on the recommendations of the manufacturer)
variable	H_2O

2. **Run a PCR under standard conditions, using an elongation temperature of 60-72°C** (depending on the recommendations of the manufacturer) **with an elongation time of 1 min/kb.**

Protocol (continued)

3. **Purify PCR product to remove *Pfu* polymerase and primers.**
If PCR reaction produced a single product of the expected size, purification using a clean-up kit to remove may be sufficient to proceed to the knockout construct generation reaction. It is, however, recommended to isolate the PCR product by preparative gel electrophoresis which may reduce efforts for identifying a correct clone after knockout construct generation reaction. Elute PCR product from purification column with water.

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4. **Determine the amount of purified PCR product by gel electrophoresis through band intensity comparison with the DNA marker or by quantification with suitable spectrophotometers.**

Both, the purification and the quantitation of the PCR product are essential, because the combinatorial cloning procedure relies on exact amounts of pure material.

Reaction for knockout construct generation

1. **Mix the following components in a reaction vessel and total volume of 25 µl**:

10 µL	pKOSG-IBA-dicty1
15 ng	PCR product 1 (LA)
15 ng	PCR product 2 (RA)
1 µL	DTT/ATP mix [250 mM DTT; 12.5 mM ATP]
1 µL	T4-DNA-Ligase [1 U/µl]
0.5 µL	*Esp3I* [10 U/µl]
variable	H_2O

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) 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.**

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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.**

Protocol (continued)

<u>Analysis of generated vectors</u>

1. **Pick 10 white colonies and perform DNA mini preparation.**
2. **Plasmids are digested with the restriction enzyme whose recognition sequence had been added to the respective LA1 and RA2 primers.**
3. **Analytical restriction digests are analyzed by agarose gel electrophoresis.**
4. **Clones with the expected band pattern are selected and sequenced using the FUSION-Primer for (5'GGGAATAAGGGCGACACGG3') and FUSION-Primer rev (5'GAGCGTCGATTTTTGTGATGC3'), that bind in the pKOSG-IBA-Dicty1 sequence upstream of the left arm and downstream of the right arm, respectively.**
5. **Plasmid maxi preparations are carried out on constructs with correct sequences. Of these, 10 µg are digested using the same restriction enzymes that linearize the plasmid, and transformed in *D. discoideum* using standard methods (see www.dictybase.org)**
6. **The BS(R) cassette can be removed after a successful knock out using the protocol published in Faix et al., 2004 (NAR).**

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Solutions
For Life Sciences

IBA Headquarters IBA
IBA GmbH
Rudolf-Wissell-Str. 28
37079 Goettingen
Germany
Tel: +49 (0) 551-50672-0
Fax: +49 (0) 551-50672-181
E-mail: info@iba-lifesciences.com

US Distribution Center
1328 Ashby Road
Olivette, MO 63132
USA
Tel. 1-877-IBA-GmbH
(1-877-422-4624)
Fax 1-888-531-6813
E-mail: info@iba-lifesciences.com