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Phosphorothioate-based BioBrick cloning (Potsdam) Standard

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1 Abstract

Cloning with restriction enzymes is frequently impaired by multiple occurrences of the desired recognition sites and often introduces unnecessary sequences as cloning artifacts. For sequences amenable to primer extension, sequence independent and seamless cloning strategies are preferred. Some are established (PCR assembly cloning, end-recession cloning/Gibson cloning) but these are often difficult to control. This RFC adapts the "Phosphorothioate-based ligase-independent gene cloning" PLICing) to BioBrick cloning with restriction sites (e.g. RFC 10) and provides a universal cloning protocol. Phosphorothioate containing oligonucleotides introduced in double-stranded DNA by extension technologies can be efficiently cleaved with iodine leaving defined 3' overhangs thus enabling seamless cloning with or without ligase. Vector amplification is avoided by the introduction of two 3'-overhang generating restriction sites (ApaI, SphI) within the pre- and suffix of e.g. RFC 10 allowing for directional cloning of iodine treated inserts in a standard vector. A standard RFC 10 vector harboring a red fluorescent protein gene flanked by ApaI and SphI provides visual control of the cloning procedure.

2 Relation to other BBF RFCs

The BBF RFC 91 expands the RFC 10 prefix and suffix.

3 Motivation

Using idempotent assembly standards e.g. according to RFC 10 is a serial process that is time and material consuming and may require the removal of interfering restriction sites from your gene of interest. Specifically in larger fragments, the occurrence of the reserved cloning sites is very likely, and site-directed mutagenesis may significantly delay a project without any scientific merit. Next to these major draw backs, serial cloning with enzymes can pose several technical annoyances such as repeated UV exposure during gel extractions or prolonged periods of time required for cell growth and plasmid amplification.

4 Method Overview

BBF RFC 91 adapts an enzyme-free cloning approach named PLICing (Blanusa *et al*., 2010) to Biobrick cloning. A new standard cloning vector for *E. coli* is described and available from the Registry of Standard Biological Parts, in which the RFC 10 prefix is extended at the 3' end by an Apa I restriction site and the RFC 10 suffix is extend at the 5' end by an Sph I restriction site. To aid in the detection of new recombinant clones, this vector may carry any type of marker. We use an RFP (red fluorescent protein) expression cassette, which gets replaced by the gene of interest (GOI) and allows visual control of successful cloning based on the loss of the red colony color. For cloning, the vector is digested with ApaI and SphI. Upon cleavage these restriction sites provide 3' overhangs.

The GOI is amplified with primers containing overhangs with phosphorothioate linked nucleotides at their 5' ends that are compatible with the ApaI and SphI overhangs of the vector. Phosphorothioate linked nucleotides are available from many vendors with only a low additional fee. These 5' phosphorothioates of the amplified GOI are then chemically cleaved using an iodine/ethanol solution leaving 3' overhangs. After that, digested backbone and the 'PLICed' GOI are ligated in the usual fashion. As there is no limit for the overhang length, which can be generated by the PLICing method, several fragments can be linked in a scar free and enzyme free one pot reaction (Marienhagen et al., 2012).

In the original PLICing method, no enzymes are used at all, because the vector is also amplified with phosphorothioate oligonucleotides. However, amplifying whole vectors is sometimes difficult and may introduce undesired errors, which are not detected by sequencing. Therefore, this standard uses additional restriction enzymes within the prevalent RFC 10 standard. The combination of both (enzyme and enzyme-free cloning) also provides the additional option to use this standard in restriction enzyme based cloning approaches.

The restriction site independent nature of the standard offers great flexibility. Overhangs for inserts can be designed such that the ApaI or SphI restriction sites are maintained or destroyed after ligation by varying the base adjacent to the four hybridizing nucleotides. Thus, this standard offers the unique opportunity to use an idempotent cloning strategy at the 5'or 3'end or even in the middle between two joined parts. In the latter case, the optional 'scar' between two parts may contain the ApaI and SphI sites.

In summary, this approach provides an assembly standard without using restriction enzymes for digestion of the GOI. Furthermore, the RFP expression cassette serves as a ligation control system in which red fluorescent colonies indicate a failed and colorless colonies a successful ligation.

5 Detailed Description

5.1 PLICing Method in general

The basis of the proposed standard is the PLICing method published by Blanusa *et al*. in 2010. This method use phosphorothioate primers for amplifying the GOI and the backbone. After cleaving the phosphorothioates, backbone and GOI can be mixed and ligated without any restriction enzyme (Fig.

1). Nevertheless, there are many problems with amplification of large backbones via PCR without any mutation. Therefore, here we define a hybrid method in which the backbone is digested with ApaI and SphI. The GOI is amplified with phosphorothioate primers, digested, mixed, and ligated with the backbone.

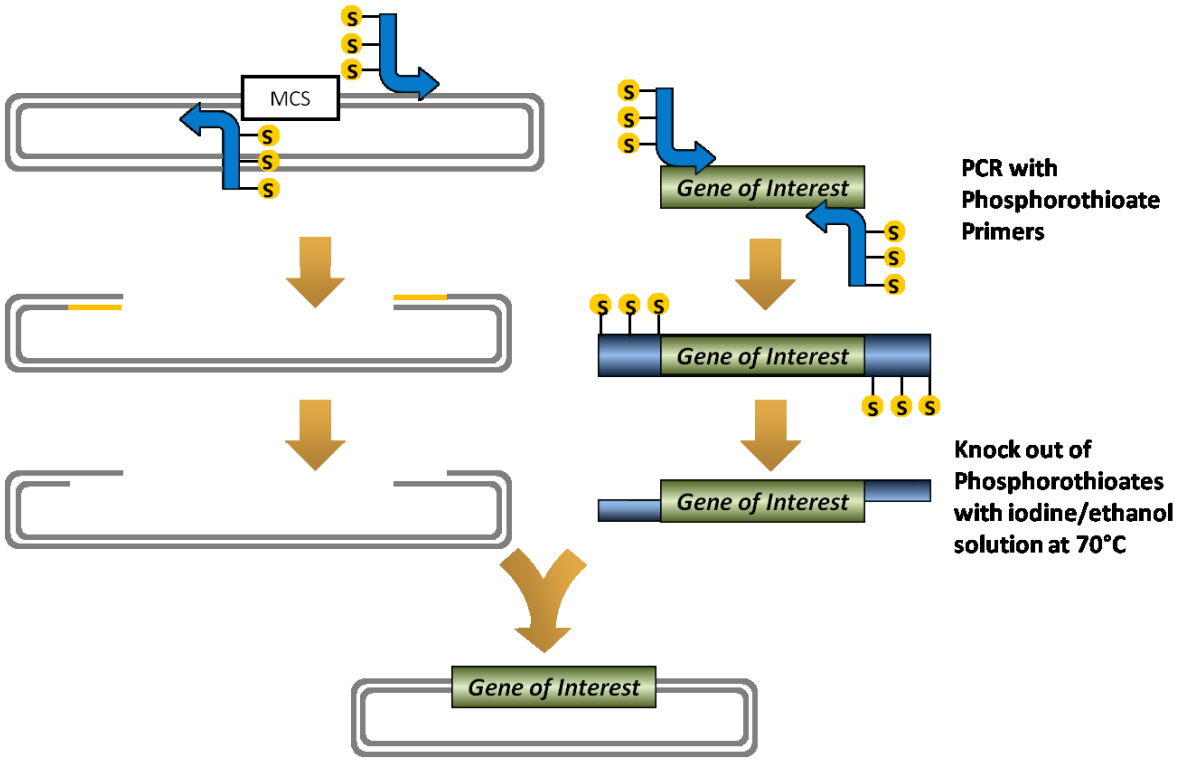

Fig. 1: PLICing method in general as published by Blanusa *et al.* in 2010

5.2 Phosphorothioat cleavage in combination with a digested vector

We demonstrated the proposed method by amplifying our gene of interest (GOI) with primers, which had a phosphorothioate overhang. In the test case, the overhang for the forward primer was GGCCT to inactivate the ApaI restriction site and CATGA for the reverse primer to inactivate the SphI restriction site after ligation. The four nucleotides of these overhangs are complementary to the digested restriction sites of ApaI and SphI in the new cloning RFP standard vector. For the PCR we used the Phusion polymerase. An 8 µl sample of the PCR product was 'pliced' with 1 µL cleavage buffer (0.5 M Tris-HCl, pH 9.0), 0.6 µl iodine solution (100 mM iodine solution in 99 % ethanol), and 0.4 µl deionized H_2O for 5 min at 70 °C using a thermocycler. After the plicing process, the insert was mixed with the digested backbone, ligated at room temperature for 1 h and directly transformed into *E. coli* (summary: Fig. 2).

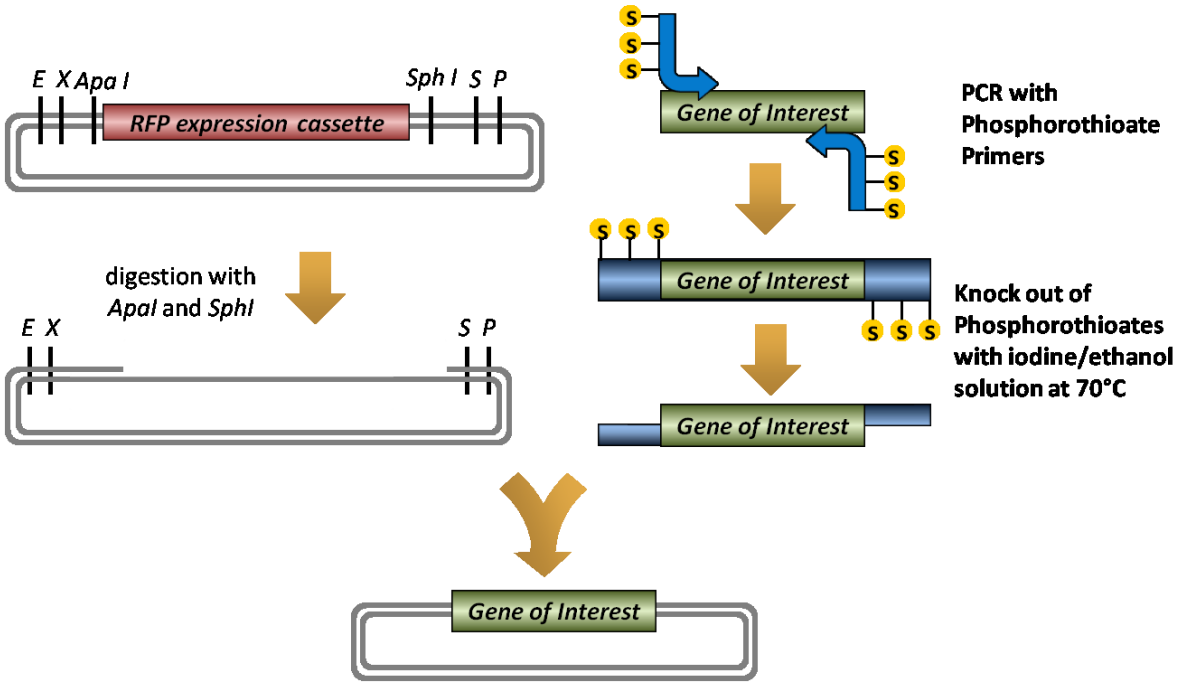

Fig. 2: Experimental Setup for BBF RFC 91using the 3' overhang generating restriction enzymes ApaI and SphI for the vector and the phosphorotioate knock-out for the insert.

To assemble several GOI, it is important to use different overhangs to ensure the right order of the GOI in the backbone. The flanking GOI must contain phosphorothioates at the 5' end that correspond to the resulting overhangs of the backbone after digestion with ApaI and SphI (Fig. 3).

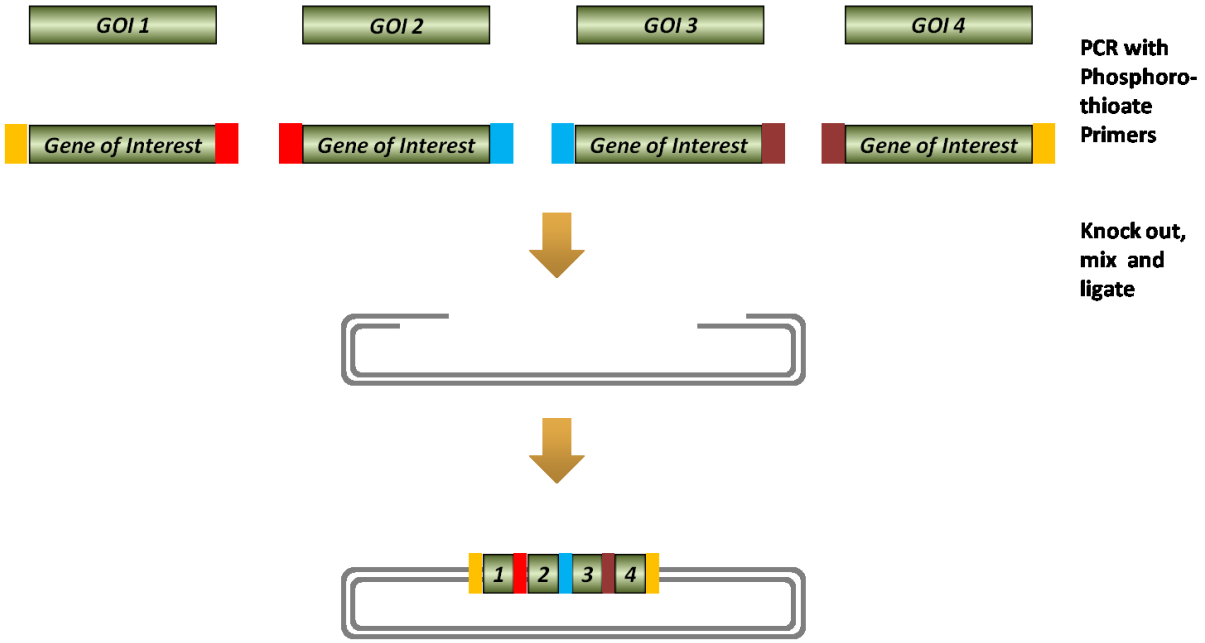

Fig. 3: Assembly of several GOI in the Potsdam standard vector. Different colors refer to different overhangs.

5.3 Experiences

5.3.1 Fluorescence of E. coli cells transformed with the Potsdam Standard RFP Cloning vector

The red fluorescence of the transformed colonies is obvious without IPTG induction of the RFP expression after 24 h incubation at 37°C followed by an overnight incubation at 8°C. To speed up the cloning, one can use an LED light to see red fluorescence even after 16 h incubation at 37°C. IPTG is not required for RFP production due to the leakiness of the upstream lac operator. However, for an overnight culture without any further incubation, we observed that it is better to incubate with IPTG. After pelleting the cells, one can see the cherry-colored cell pellets. Whether IPTG can be added or not depends on the cloned insert.

5.3.2 Using Potsdam Standard RFP Cloning vector as ligation control

We tested different combinations to assess the efficiencies of different experimental designs. Four conditions were tested: the ligation of the pliced GOI with the unpurified digested backbone with and without ligase, and the ligation of the pliced GOI with the purified digested backbone with and without ligase. After transformation, we picked only clones that had lost their red fluorescence indicating that the ligation was successful. To quantify the results, we counted the red colonies as well as the colonies without red fluorescence. The ligation with the electrophoretically purified digested backbone was as expected more efficient than without any purification. Interestingly, it turned out that the use of ligase is not necessary for the ligation process under these conditions and the relative number of positive clones remained high permitting ligation-free cloning with the short overhangs albeit with a low total efficiency.

5.4 Restriction Enzymes

5.4.1 Apa I

Restriction site

5'…G G G C C|C …3`
3'…C|C C G G G …5'

Vendors

Bangalore Genei, EURx Ltd., Invitrogen Cooperation, Molecular Biology Resources – CHIMERx, New England Bioloabs, Nippon Gene Co, Ltd., , Promega Corporation, Obiogene, Roche Applied Science, SibEnzyme Ltd., Sigma Chemical Corporation, Takara Bio Inc., Thermo Scientific Fermantas, Vivantis Technologies.

Isoschizomers

Bsp120I, PspOMI.

Activity in NEB buffers

NEB 1: 25 %; NEB 2: 50 %; NEB 3 0 %; NEB 4 100 %

Activity at 37 °C

100%

Heat Inactivation

65 °C for 20 minutes

Methylation sensitivity

not sensitive to *dam* methylation

5.4.2 Sph I

Restriction site

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5'…G C A T G|C …3`
3'…C|G T A C G …5'
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Vendors

EURx Ltd., Invitrogen Cooperation, Minotech Biotechnology, Molecular Biology Resources – CHIMERx, New England Bioloabs, Nippon Gene Co, Ltd., Promega Corporation, Obiogene, Roche Applied Science, SibEnzyme Ltd., Sigma Chemical Corporation, Takara Bio Inc., Toyobo Biochemicals, Vivantis Technologies.

Isoschizomers

BbuI, PaeI

Activity in NEB buffers

NEB 1 100 %; NEB 2 100 %;NEB 3 50 %; NEB 4 100 %

Heat Inactivation

65 °C for 20 minutes

Methylation sensitivity

not sensitive to *dam, dcm* and CpG methylation

6 Copyright Notice

7 References

- Blanusa M, Schenk A, Sadeghi H, Marienhagen J, Schwaneberg U., “Phosphorothioate-based ligase-independent gene cloning (PLICing): An enzyme-free and sequence-independent cloning method.” Anal Biochem, 2010 Nov 15;406(2):141-6.
- Liu XP, Liu JH, “The terminal 5' phosphate and proximate phosphorothioate promote ligation-independent cloning.” Protein Sci. 2010 May;19(5):967-73.
- Marienhagen J, Dennig A, Schwaneberg U. “Phosphorothioate-based DNA recombination: an enzyme-free method for the combinatorial assembly of multiple DNA fragments.” Biotechniques Rapid Dispatch, 2012:1-6, doi: 10.2144/000113865.