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Small heat shock proteins

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CHAPTER 2

A PCR amplification strategy for unrestricted generation of chimeric genes

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Abstract

For analyzing protein function, protein dynamics or protein-protein interactions, the use of chimeric proteins has become an indispensable tool. The generation of DNA constructs coding for such fused proteins can, however, be a tedious process. Currently used strategies often make use of available endonuclease restriction sites leading to limitations in the choice on the site of fusion between two genes and problems on how to maintain protein secondary structures. We have developed a cloning strategy to get around these disadvantages that is based on a single round of PCR amplification followed by antibiotic resistant gene complementation.

Introduction & results

Methods for creating either tagged or chimeric proteins usually depend on a combination of PCR amplification of the gene of interest followed by endonuclease mediated cloning into a plasmid. The generation of chimeric genes is often performed using a combination of available endonuclease sites (1) or the introduction of required endonuclease sites (2). Although often efficient, especially the choices at which site in the gene a chimeric gene can be produced is restricted by the presence of endonuclease recognition sites and often requires the introduction of additional bases. Also, several rounds of PCR amplification and cloning are required before a desirably construct is obtained. Furthermore, cloning of genes in frame with for instance, a fluorescent protein encoding gene, always introduces additional aminoacids between both proteins originating from the multiple cloning site of the plasmid used.

Here we report on the design of a cloning strategy which, independently of the absence or presence of specific endonuclease recognition sites, allows for fast and reliable generation of chimeric genes in one single round of PCR amplification, without the introduction of additional aminoacids between the fused proteins. Furthermore, our strategy allows to freely choose where to make a fusion between two genes, thus providing means to avoid disruption of secondary protein structure. In addition, it utilizes antibiotic resistant gene complementation to select for the correct fusion construct in E. coli. This results in a 100% efficiency in obtaining the correct ligated product.

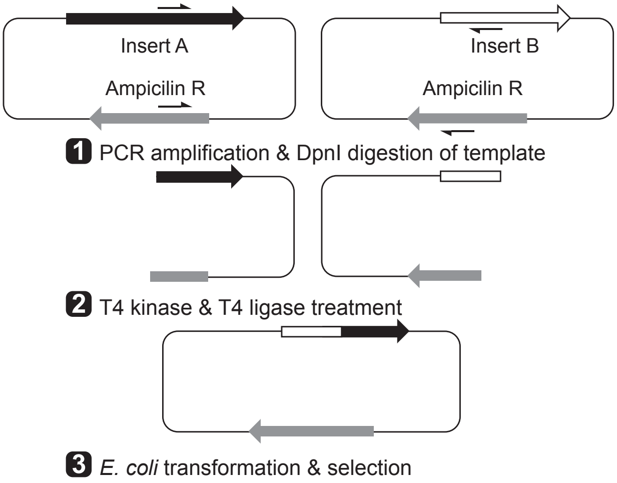
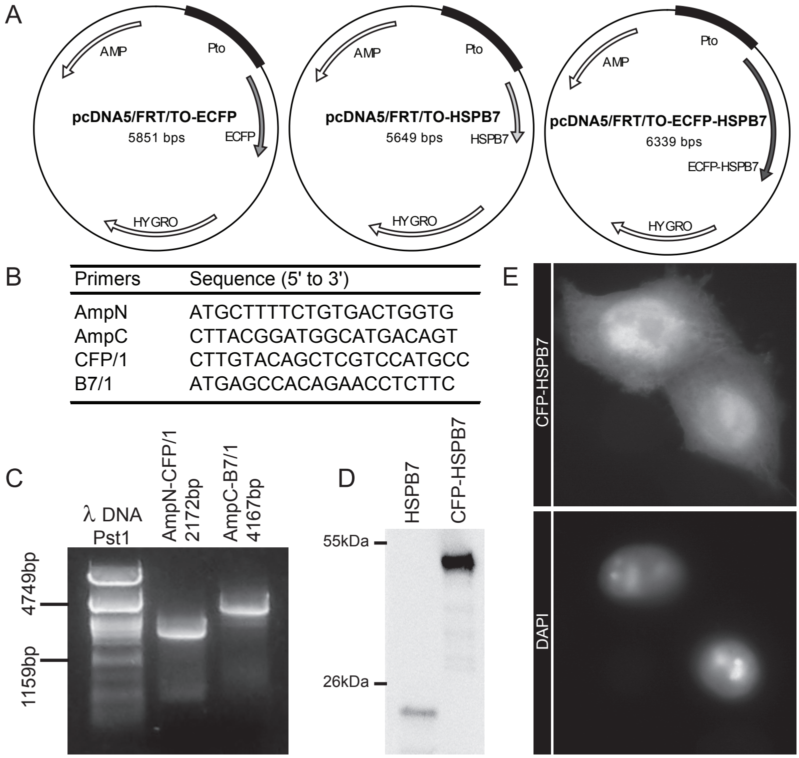

Insert A
Ampicilin R
Insert B
Ampicilin R
1 PCR amplification & DpnI digestion of template
2 T4 kinase & T4 ligase treatment
3 *E. coli* transformation & selection

Figure 1. Schematic outline of the strategy for generating chimeric genes. After a single round of PCR amplification of Insert A or Insert B together with the N-terminal or C-terminal part of the Ampicillin gene respectively, methylated DNA template was removed by DpnI digestion (1). Secondly, both fragments are combined and treated with T4 kinase and T4 ligase (2). Selection of the correctly ligated product is done in E. coli by exploiting the formation of a correct resistance gene and hence acquired antibiotic resistance (3).

Our strategy (Figure 1) requires the availability of two plasmids with the same backbone, one containing gene A and the other gene B or a tag. In the first step sequence A is amplified together with the disired part of the vector backbone including half of the Ampicillin resistance gene. The other part for the fusion plasmid is amplified containing the desired part of sequence B together with the vector backbone including the complementing half of the Ampicillin resist-


A
AMP
Pto
pcDNA5/FRT/TO-ECFP
5851 bps
ECFP
HYGRO
AMP
Pto
pcDNA5/FRT/TO-HSPB7
5649 bps
HSPB7
HYGRO
Pto
AMP
pcDNA5/FRT/TO-ECFP-HSPB7
6339 bps
ECFP-HSPB7
HYGRO
B
C
λ DNA Pst1
AmpN-CFP/1 2172bp
AmpC-B7/1 4167bp
4749bp
1159bp
D
HSPB7
CFP-HSPB7
55kDa
26kDa
E
CFP-HSPB7
DAPI

Primers	Sequence (5' to 3')
AmpN	ATGCTTTTCTGTGACTGGTG
AmpC	CTTACGGATGGCATGACAGT
CFP/1	CTTGTACAGCTCGTCCATGCC
B7/1	ATGAGCCACAGAACCTCTTC

Figure 2. Fusion of HSPB7 with ECFP and expression in HELA S3 cells. To generate a fusion between ECFP and HSPB7 (panel A), primers were selected as indicated in panel B. The PCR products (panel C) were ligated and transfected in HELA S3 cells. After 48hrs, protein expression was analyzed by Western-blotting (panel D) using monoclonal HSPB7 antibody (Abnova). The fusion protein was visualized using fluorescent microscopy (panel E), showing localization in both the cytosol and the nucleus. The nucleus was visualized using DAPI staining.

ance gene. Next, both fragments are digested for two hours with DpnI to allow for methylated template removal from the PCR reaction. This effectively reduces the chance of original vector contamination during E. coli transformation. Then, both fragments are mixed and treated with T4 kinase and T4 ligase for two hours. Selection for the correct ligated product is achieved by the functional restoration of the bacterial resistance gene. This leads to a 100% efficiency in obtaining clones harboring the correctly fused sequences. The same process can be performed with plasmids containing any other bacterial selection gene. As the choice for primer binding sites on both gene A and gene B is unrestricted, a chimeric gene can be generated at any place so that disruption of secondary protein structure can be avoided.

As an example, we generated a fusion between the Enhanced Cyan Fluorescent Protein (ECFP) and HSPB7, a small heat shock protein. The plasmids used were pcDNA5/FRT/TO-ECFP and pcDNA5/FRT/TO-HSPB7. Primers used for PCR on pcDNA5/FRT/TO-ECFP were AmpN and CFP/1 lacking the stop codon. For pcDNA5/FRT/TO-HSPB7, primer AmpC was used in combination with B7/1 (Figure 2a,b). The PCR reactions were carried out in a total volume of 50uL containing 10-30 ng plasmid template, 200 μM of each dNTP, 5 μL Pfuturbo buffer, 30 pmol of each primer, 3 μL DMSO and 2,5U of Pfuturbo DNA polymerase (Stratagene). The amplification

reaction was started with a 1 minute incubation at 95 ^{0}C followed by 19 cycles of denaturation at 95 ^{0}C for 50 seconds, annealing at 55 ^{0}C for 50 seconds and amplification at 68 ^{0}C for 1 minute per kb. The reaction was ended by a final incubation step for 8 minutes at 68 ^{0}C.
After amplification, the fragments (Figure 2c) were treated with DpnI for two hours and ligated at room temperature for two hours using 5U of T4 DNA ligase in the presence of 10U T4 kinase buffered by T4 DNA ligase buffer (Invitrogen). Several clones were selected for restriction analysis and sequenced, all of which were correct. Transfection of the new fusion plasmid in mammalian cells clearly shows correct expression of the fusion protein by Western blot analysis (Figure 2d). This construct was effectively used to visualize ECFP-HSPB7 in HELA cells using fluorescent microscopy (Figure 2e).

In conclusion, our strategy allows for fast and efficient generation of chimeric genes or tagged genes using an internal selection for appropriately ligated fragments of a functional bacterial resistance gene. Furthermore, it circumvents the need for introducing appropriate endonuclease recognition sequences and allows for the fusion of two sequences taking into account the preservation of secondary protein structures.

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