



1.2 PCR mutagenesis techniques and RTS

1.2.1 PCR mutagenesis and direct expression

Now that the post-genomic era has begun, many scientists are using the enormous amount of sequence data gathered by the genome projects for protein expression studies. Studying structure-function relationships of these proteins often involves mutating their genes.

The polymerase chain reaction (PCR) has proven to be particularly useful for a wide range of mutation procedures and applications. PCR mutagenesis procedures make it possible to modify and engineer any target DNA easily and efficiently. This includes the introduction of:

- ▶ Point mutations
- ▶ Deletions or insertions
- ▶ Domain fusion
- ▶ Random mutagenesis

The RTS *E. coli* Linear Template Generation Sets, which generate linear templates for direct expression in RTS 100 *E. coli* HY reactions, are designed to be used in PCR. Therefore, the use of these sets can easily be combined with established PCR mutagenesis techniques. The sets allow simultaneous introduction of mutations along with the regulatory elements needed for RTS expression.

We describe here a technique for introducing point mutations and fusing domains while generating linear expression templates. The whole process, involving two PCR steps and *in vitro* expression can be completed in less than 16 hours. Since it was not produced *in vivo*, the expressed protein can be tested directly; it does not need to be purified from a complex *E. coli* cell. Other procedures, *e.g.*, generation of deletions or insertions, can easily be done by the same technique (see the PCR literature, *e.g.*, McPherson and Møller, 2000; Newton and Graham, 1997).

1.2.2 Introduction of point mutations into linear expression templates

The basic procedure that was adapted for introduction of point mutations is called “two-sided splicing by overlap extension” (Horton et al., 1989). The principle of this efficient and specific method is shown in Figure 11.

In the first step, two separate PCRs are used to generate the primary PCR products, designated PCR 1a and PCR 1b. Both primary PCR products contain the desired mutation as well as overlap regions that are attached to the beginning and end of the coding sequence. These overlaps are needed for production of the linear expression template in the second PCR. PCR 1a is performed with primers 1 and 2; PCR 1b is performed with primers 3 and 4 (Figure 11). The mutation is introduced via primers 2 and 3, while primers 1 and 4 are used to attach the overlap regions. To design primers 1 and 4, follow the principles for designing gene-specific primers outlined in the Instruction Manuals for the RTS *E. coli* Linear Template Generation Sets.

The products from the first PCRs (fragments 1a and 1b) must be gel purified to separate them from wild-type template DNA. The products can then be used, in equal amounts, as templates for the second PCR.

Fusion of the two mutated fragments and addition of all regulatory elements can easily be done in a single PCR. In the first cycle of the second PCR, the two primary PCR products containing the mutation within complementary tails anneal and are extended to form one full-length mutated fragment (Figure 11). Then DNA fragments (coding for the T7 regulatory elements and a tag), together with the outer primers (primers 5 and 6 in Figure 11) from a RTS *E. coli* Linear Template Generation Set, are added to the reaction. During subsequent PCR cycles, these DNA fragments introduce the regulatory elements necessary for expression in RTS.

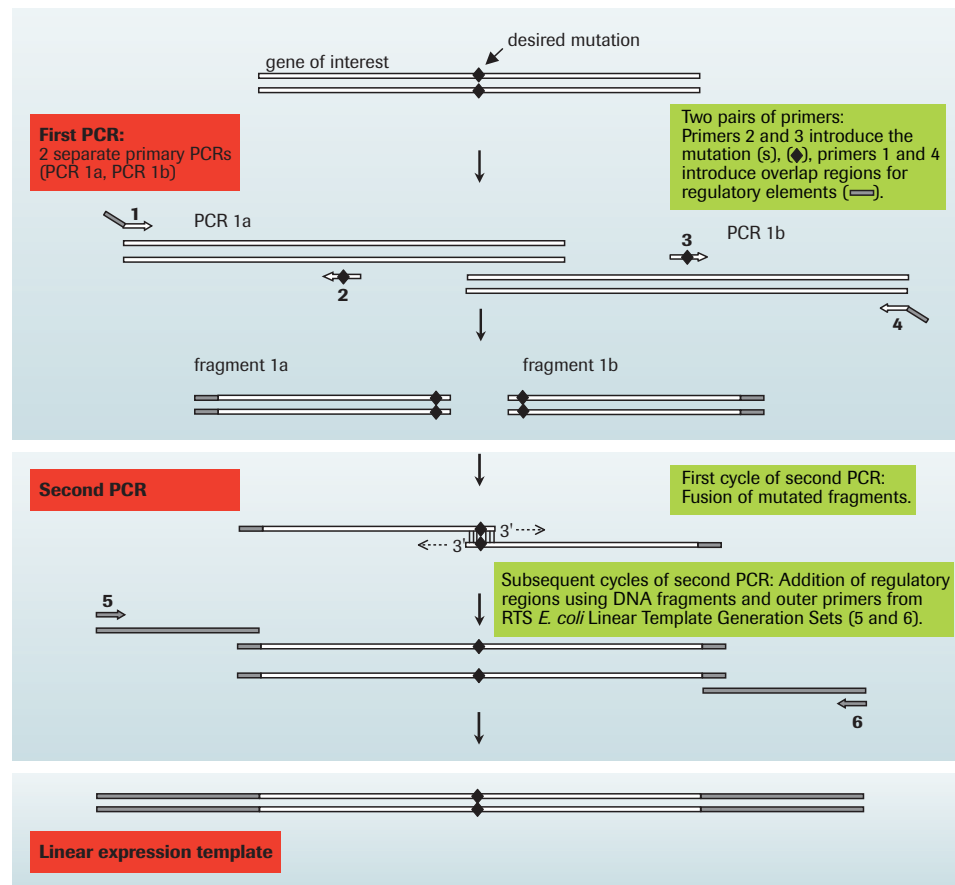


Figure 11: Introduction of point mutations combined with the generation of a linear expression template.

The first PCR (two parallel reactions, PCR 1a and PCR 1b) introduces the point mutation, the second PCR generates the linear expression template via overlap extension PCR, using the RTS *E. coli* Linear Template Generation Sets.

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1.2.2.1 Example

Influence of threonine 203 on the activity of GFP

The following experiment shows the influence of a specific amino acid (threonine 203) on the fluorescence activity of green fluorescent protein (GFP), which was described by Ormo et al. (1996). For this purpose, a single DNA triplet was mutated, causing threonine 203 to be replaced by tyrosine.

The mutation primers (primers 2 and 3, as in Figure 11) were each 26 bp long, complementary to each other, and carried the mutation in the middle part of the oligonucleotide sequence. The two primary PCRs (1a and 1b, Figure 11) were performed under standard conditions as described in the Instruction Manuals for the RTS *E. coli* Linear Template Generation Sets.

Both PCR products (Figure 12A) were purified by gel extraction and added to the second PCR in equal amounts. The full-length fragment with the mutation was then synthesized with primers 1 and 4.

In another experiment, the mutated PCR fragments were fused and regulatory elements were added to the product in a single PCR, as described in Figure 11.

As shown in Figure 12B, both reactions gave single product bands and similar yields. The size difference of the products clearly demonstrates that the second approach successfully added the regulatory elements.

This experiment shows that it is possible to fuse the two primary products and add the regulatory regions in a single reaction; it is not necessary to perform two successive PCRs. The reliability of the method was confirmed by sequencing the final PCR product. No background from the wild-type sequence was detected.

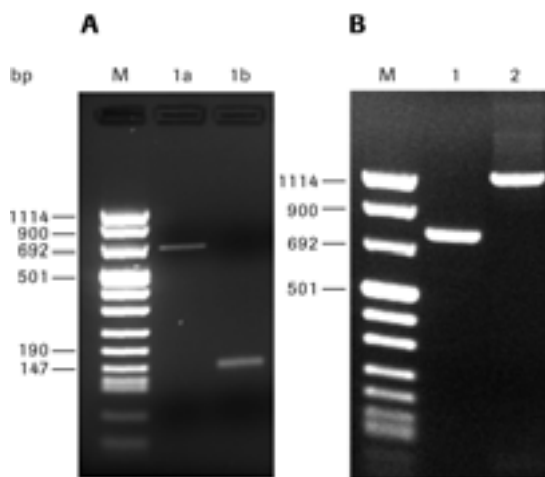


Figure 12: Introduction of a point mutation in the coding sequence of GFP combined with the generation of linear expression templates. Three μ l of the purified primary PCR products (A) and the products of the second PCR (B) were separated on 1% agarose gels. Fusion of the two mutated fragments was performed either with or without simultaneous introduction of the regulatory elements and a C-terminal His₆-tag from the RTS *E. coli* Linear Template Generation Set, His₆-tag. M: Molecular Weight Marker VIII (Roche Applied Science); 1a: DNA fragment from PCR 1a; 1b: DNA fragment from PCR 1b; 1: DNA fragment fused and amplified in a second PCR without use of RTS *E. coli* Linear Template Generation Set, His₆-tag; 2: DNA fragment 1a and 1b fused and all regulatory sequences added by RTS *E. coli* Linear Template Generation Set, His₆-tag.

The GFP mutant protein was obtained by expressing the linear template in RTS 100 *E. coli* HY. The wild-type protein was expressed in the same way. Both proteins were produced in comparable amounts as demonstrated by Western blotting (Figure 13A). In contrast to the wild-type protein, the mutant protein showed little fluorescence activity (Figure 13B), demonstrating that threonine 203 is crucial for GFP activity.

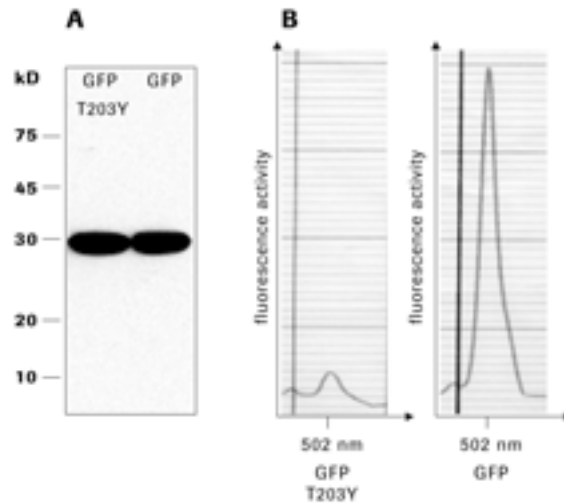


Figure 13: Expression of GFP variants.

GFP and mutated GFP (T203Y) were expressed in RTS100 *E. coli* HY from linear templates.

A: 0.5 μ l of each reaction solution were separated by SDS-PAGE, blotted and detected with Anti-His₆ Peroxidase conjugate.

B: Fluorescence activity was measured by exciting the protein at 395 nm and monitoring the emission at 430-580 nm. The reaction solutions were diluted 1:80.

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1.2.3 Fusion of domains and linear expression templates

The method described here can be used to generate a fusion protein from two separate proteins or domains. The technique joins the coding sequences and simultaneously generates linear expression templates. This method can also be used to fuse a sequence coding for a linker peptide or a random sequence to the gene of interest.

Like the method described above, this technique is based on “two-sided splicing by overlap extension” (Horton et al., 1989). The adapted procedure is shown in Figure 14. The fragments coding for the single domains or proteins are generated in two separate primary PCRs. The inner primers (2 and 3) for the primary PCRs contain a 20 bp complementary region that allows the fusion of the two domain fragments in the second PCR. The outer primers 1 and 4 carry overlap regions which introduce regulatory elements in the second PCR.

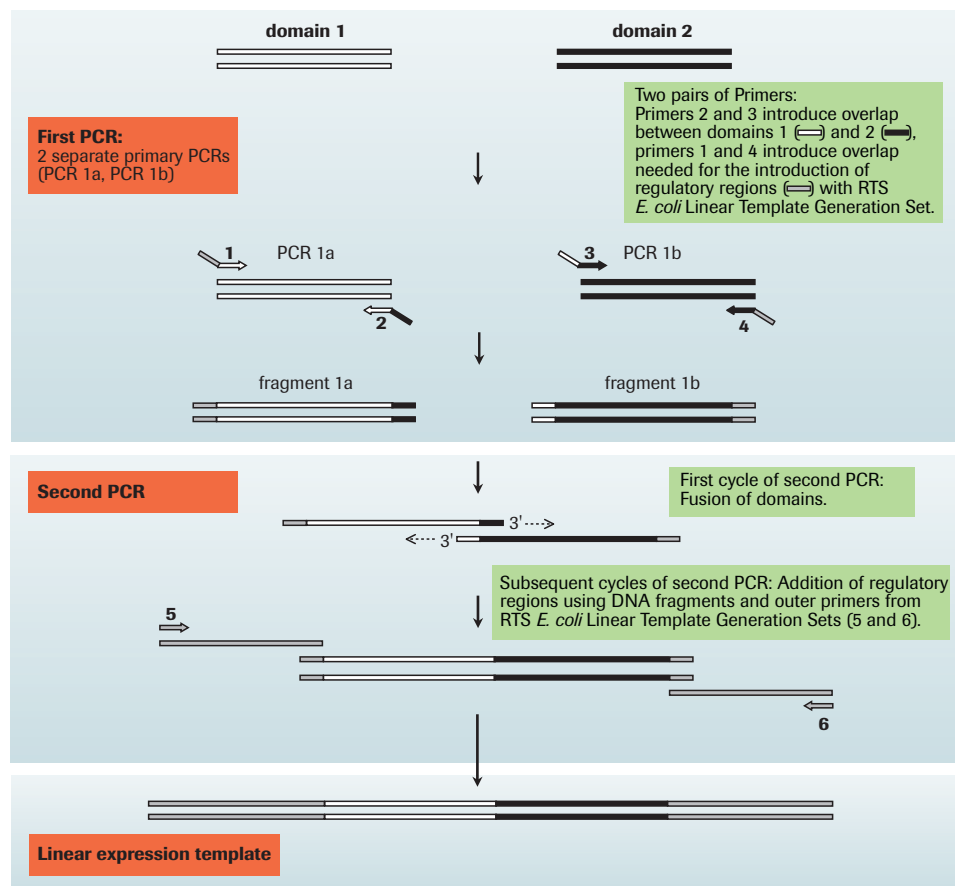


Figure 14: Scheme of domain fusion by overlap extension PCR using the RTS *E. coli* Linear Template Generation Set.

Two primary PCR products (1a and 1b) are synthesized; they have compatible ends. These fragments are joined in a second, overlap extension PCR, which also simultaneously introduces the regulatory elements necessary for expression.

1.2.3.1 Application

Two gene fragments (α and γ) were joined with the active domains of two metabolic enzymes (β and δ), as schematically illustrated in Figure 15A. The products of the two primary PCRs (Figure 15B) were added to the second PCR either before or after they were purified by gel extraction. As shown in Figure 15C and 15D, the product bands obtained with purified fragments were more specific. This experiment indicates that highly amplified, unpurified primary PCR products may be used as templates in the second PCR, but purified fragments will give more reliable results.

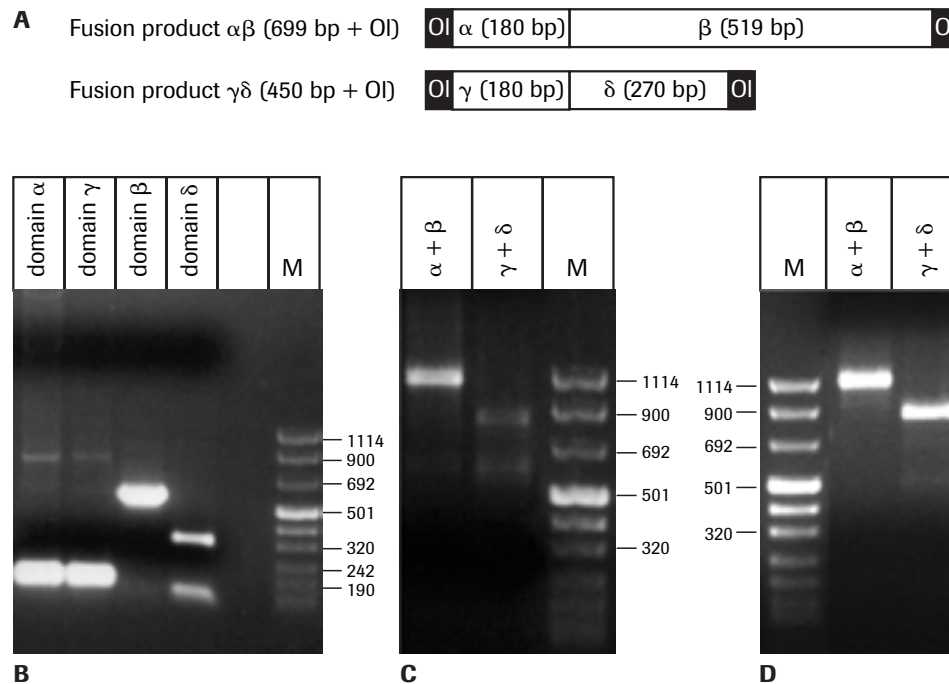


Figure 15: Generation of linear expression templates and simultaneous fusion of protein domains.

A: Schematic view of the fusion genes. **B:** Products of the first PCR (10 μ l) are separated by agarose gel electrophoresis (1%) and visualized by ethidiumbromide stain. **C:** Products of the second PCR (3 μ l) are separated by agarose gel electrophoresis (1%) and visualized by ethidiumbromide stain, unpurified product from first PCR was used as template. **D:** Products of the second PCR (3 μ l) are separated by agarose gel electrophoresis (1%) and visualized by ethidiumbromide stain, purified product from first PCR was used as template. ol: Overlap region necessary for the introduction of the regulatory elements and a C-terminal His₆-tag; M: Molecular Weight Marker VIII (Roche Applied Science).

Both fragments were used as templates in RTS 100 *E. coli* HY expression reactions. Both fusions were expressed as full-length proteins with high yield. Since the C-terminal fusion partners were active domains of metabolic enzymes, expression was monitored directly by assaying aliquots of the reaction solution for the metabolic activity (data not shown).

1.2.4 Random mutagenesis techniques

Procedures similar to those described above can be used to perform random mutagenesis, PCR misincorporation procedures and recombination strategies with the RTS *E. coli* Linear Template Generation Sets. These involve first mutating or shuffling the gene of interest, then constructing an expression template by introducing the overlap regions. In contrast to the methods described above, the product of these PCR techniques is not a single sequence, but rather a mixture of different sequences. Therefore, the constructs must be cloned into a PCR cloning vector to separate this product pool into unique species before each can be screened. Although this step requires transformation of an *E. coli* strain and plasmid preparation, it still has advantages over *in vivo* expression. In particular, the RTS reactions are more easily quantified and give more consistent results. In addition, it is not necessary to grow and lyse *E. coli* cultures to obtain the protein of interest.