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## Cloning methods

### Blunt end cloning

Blunt end cloning is one of the simplest strategies for the insertion of a gene into an expression vector. The plasmid is digested with a restriction enzyme that leaves blunt ends, and then purified by preparative gel electrophoresis, in order to remove uncut plasmid, because restriction digestion usually isn't 100 % complete. The digested plasmid furthermore needs to be dephosphorylated, in order to prevent recircularization of plasmids without insert in the following ligation step. The insert can be produced by PCR using a DNA polymerase that leaves blunt ends. If a polymerase that results in 3' deoxyadenosine overhangs is used, the ends can be polished by T4 DNA polymerase (exonuclease activity) to produce blunt ends (Koehl et al., 2003).

The blunt end inserts can also be produced by cutting them out of a vector, using restriction enzymes that produce blunt ends. In preparation for the ligation step, inserts that have been produced by PCR need to be phosphorylated. Linearized, blunt end vector and blunt end insert are then combined and incubated with T4 DNA ligase in appropriate buffer in order to form phosphodiester bonds between insert and vector.

Relatively simple construct design and cloning procedure are advantages of blunt end cloning. Disadvantages include non-directionality (the gene of interest may be ligated in the correct or inverted orientation), relatively low cloning efficiency, need for plasmid dephosphorylation to prevent circularization of vectors without insert, and the risk of inserting several inserts rather than one.

Despite the numerous disadvantages, blunt end ligation, when used in combination with end polishing by T4 DNA polymerase, has in at least one case been reported to be more efficient than sticky end ligation, because non-polished sticky ends may not always have the expected overhangs, due to restriction enzyme star activity, or because of endonuclease activity after restriction endonuclease digestion (Koehl et al., 2003).

### Sticky end cloning

The classical strategy for the insertion of DNA fragments into plasmids uses type II restriction enzymes and DNA ligase. The first experiments of this type were carried out in the early 1970's with the restriction enzyme *EcoRI*. The gene of interest can be obtained directly from plasmid DNA, by digestion with a restriction enzyme that produces defined cohesive ends, i.e. *EcoRI*. Alternatively, the DNA region of interest can be amplified by PCR with primers that contain restriction enzyme recognition sequences in non-annealing 5' tails. An additional 3 to 10 basepairs 5' of the restriction site ensure effective digestion. Theoretically, 3 extra basepairs should be sufficient for most restriction enzymes (Zimmermann et al., 1998). In real experiments however, up to 10 additional basepairs have shown to be necessary. Digestion with a single restriction enzyme that creates identical cohesive ends allows recircularization of the plasmid and leads to non-directional insertion of one or several DNA fragments. Recircularization is possible because 5'-phosphate and 3'-hydroxyl remain on DNA ends after cleavage with typeII restriction enzymes. Recircularization of plasmids that do not contain the desired insert can be avoided by a dephosphorylation step. Dephosphorylated plasmid and insert (still phosphorylated) are purified by gel electrophoresis, mixed and incubated with DNA

ligase, leading to covalent attachment of the insert to the vector via phosphodiester bonds.

Efficiency of this method can be improved by digesting with two restriction endonucleases that produce different sticky ends. Recircularization of plasmids that have been digested with both enzymes is not possible, because the ends of the plasmids are not compatible. Furthermore, the gene of interest can only be inserted in the desired orientation.

Directionality can also be achieved by cutting with a single restriction enzyme that recognizes a variety of sequences, so that two different cohesive ends can be created by digestion with a single enzyme. EcoRII, for instance, can recognize both, CCAGG and CCTGG and leaves sticky ends. If these two sequences are used for cloning into an expression vector, then cleavage with a single enzyme will still allow directional cloning and prevent recircularization of the plasmid. Enzymes of this type include EcoRII, AccI, Aval, HaeII and HindII.

The gene of interest may in some cases contain an internal recognition sequence for the restriction enzyme that would be required for a specific cloning experiment. In such a case, the insert cannot be prepared by digestion with the restriction endonuclease. The problem can be circumvented by preparing the insert by sticky-end PCR.

### **TA cloning**

TA cloning is a very simple method that allows nondirectional cloning of PCR products. The gene of interest is PCR amplified, using Taq DNA polymerase. Due to the terminal transferase activity of this non-proofreading enzyme, the PCR product obtains single deoxyadenosine 3' overhangs that can be cloned into vectors containing single 3' deoxythymidine overhangs. T-vectors can be prepared from any vector that has been digested with a restriction enzyme that produces blunt ends, by incubation with Taq polymerase in the presence of dTTP, but in the absence of all other nucleotides (Marchuk et al., 1991).

### **Sticky end PCR**

Digestion of inserts by restriction enzymes is not always efficient, even if the number of extra basepairs flanking each restriction site is theoretically sufficient. The ends that are removed from the PCR product by restriction enzyme digestion are very small, and the change in size of the DNA fragment is difficult to see on a regular agarose gel (Zimmermann et al., 1998). If digestion fails, ligation will also fail, yet the cause for failing ligations is often unclear in real experiments. A further problem that can arise is that the gene of interest may contain one or more internal recognition sites for the same restriction endonuclease that is to be used for cloning.

All mentioned problems can be solved by using sticky end PCR. Sticky end PCR (Zeng, 1998) is an alternative method for the production of inserts with the correct overhangs for ligation, without the use of restriction enzymes. Two separate PCR reactions are carried out, each leading to PCR products with the same core, but with an offset of several base pairs at the ends. The correct overhangs are obtained by combining the two PCR products, heating them to 98° C for a few seconds, and cooling them down to allow annealing. 25 % of the product then contains insert DNA with the desired flanking regions for ligation. The vector can be classically digested with restriction enzymes.

The same basic strategy has also been published under the name “hetero-stagger cloning” (Liu, 1996) Figure 3 illustrates the principle behind sticky end PCR.

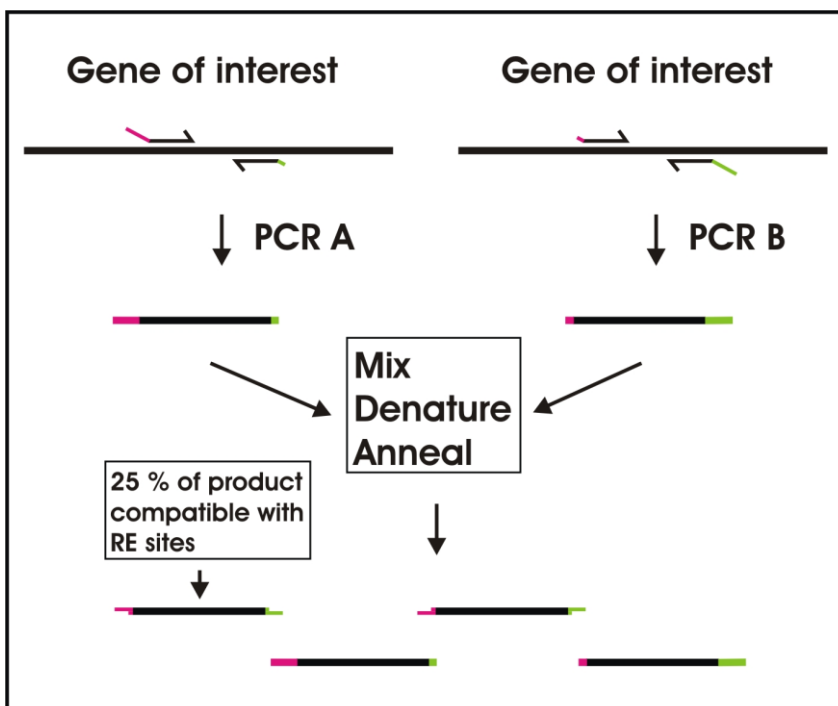


Figure 3) A schematic representation of sticky end PCR. Double stranded DNA is represented as a wide single line. The DNA region of interest is amplified by PCR. Two PCR reactions use primers with the same annealing regions, but different 5' tails, leading to a PCR product with the same core, but a slight offset at the ends. Heat denaturation and reannealing of a mixture of the two PCR products leads to 25 % of product with the correct cohesive ends for sticky end ligation into a restriction enzyme digested plasmid.

### Polymerase recombination

Two linear DNA fragments, i.e. PCR products, that contain sufficient overlap for annealing to each other, can be combined by PCR (Yolov and Shabarova, 1990). The two DNA fragments are used as templates. The primers are designed to anneal to the free ends of the DNA fragments (the ends that do not contain the overlap). The result is a linear polymerase reaction leading to two single stranded products that are partial, opposite strands of the final product, and that can anneal to each other at the 3' ends. The missing strands are synthesized by DNA polymerase, using the 3' ends of the single stranded DNA fragments as primers. Once the full-length product has been synthesized, it serves as a template, leading to exponential PCR amplification.

A schematic representation of polymerase recombination is shown in Figure 4.

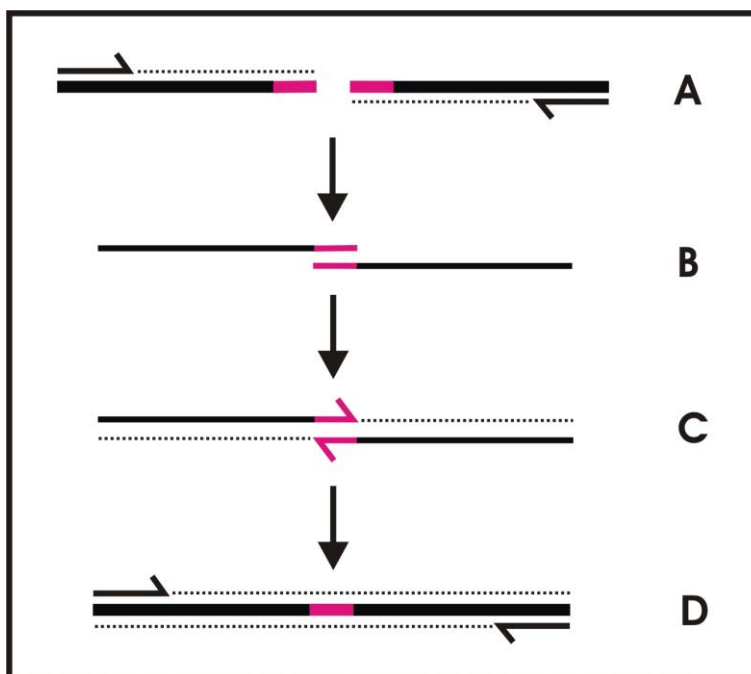


Figure 4) A schematic representation of Polymerase recombination. Double stranded DNA is shown as a single wide line. Arrows indicate the direction of primer elongation. Dotted lines represent the strands that are synthesized in the reaction. The overlap region between the two DNA fragments is shown in magenta. A: Primers produce single stranded DNA in a linear reaction. B: The two single stranded DNA fragments anneal to each other at the overlap region. C: the 3' ends of DNA fragments serve as primers for the filling in of the missing strands. D: The desired double stranded, full-length DNA fragment is now present in solution and serves as a template for PCR amplification.

### Integration PCR

Integration PCR can be used for the seamless integration of any gene of interest at any position of a plasmid. The principle is basically the same as for QuikChange<sup>®</sup> site directed mutagenesis, except that the integrated DNA fragment is much larger, and that the mutagenesis primers are produced in a first PCR reaction. The method has been used by scientists for several years. Seamless cloning is possible, without the use of restriction enzymes (with the exception of Dpn1) and ligase. In the first step, the gene of interest is amplified by PCR, using primers that contain nonannealing 5' tails that are complementary to the desired site of integration of the expression plasmid (Chen et al, 2000; Geiser et al., 2001). The tails should be approximately 20 bp in length, or have a melting temperature that is high enough for annealing in the second PCR step. The PCR product is then purified and used as a "megaprimer" in a second round of PCR that uses the expression plasmid as template. The megaprimer anneals to the template via the 20 nt complementary region on each side of the gene of interest. Pfu Polymerase is used to amplify the full expression plasmid, resulting in the integration of the gene of interest. Elongation has to be carried out at 68° C, because Pfu Polymerase (Stratagene) does not strand displace at this temperature. The PCR product is then incubated with DpnI, which digests methylated template DNA produced in dam<sup>+</sup> *E. coli* strains, but not the non-methylated PCR products. The end product is then directly used to transform competent *E. coli* cells. Sense and antisense strands of the product from the second PCR have complementary, overlapping, single stranded ends that are long enough to allow transformation without ligation (strong hydrogen bonding). Figure 5 shows a schematic diagram illustrating the principle behind integration PCR.

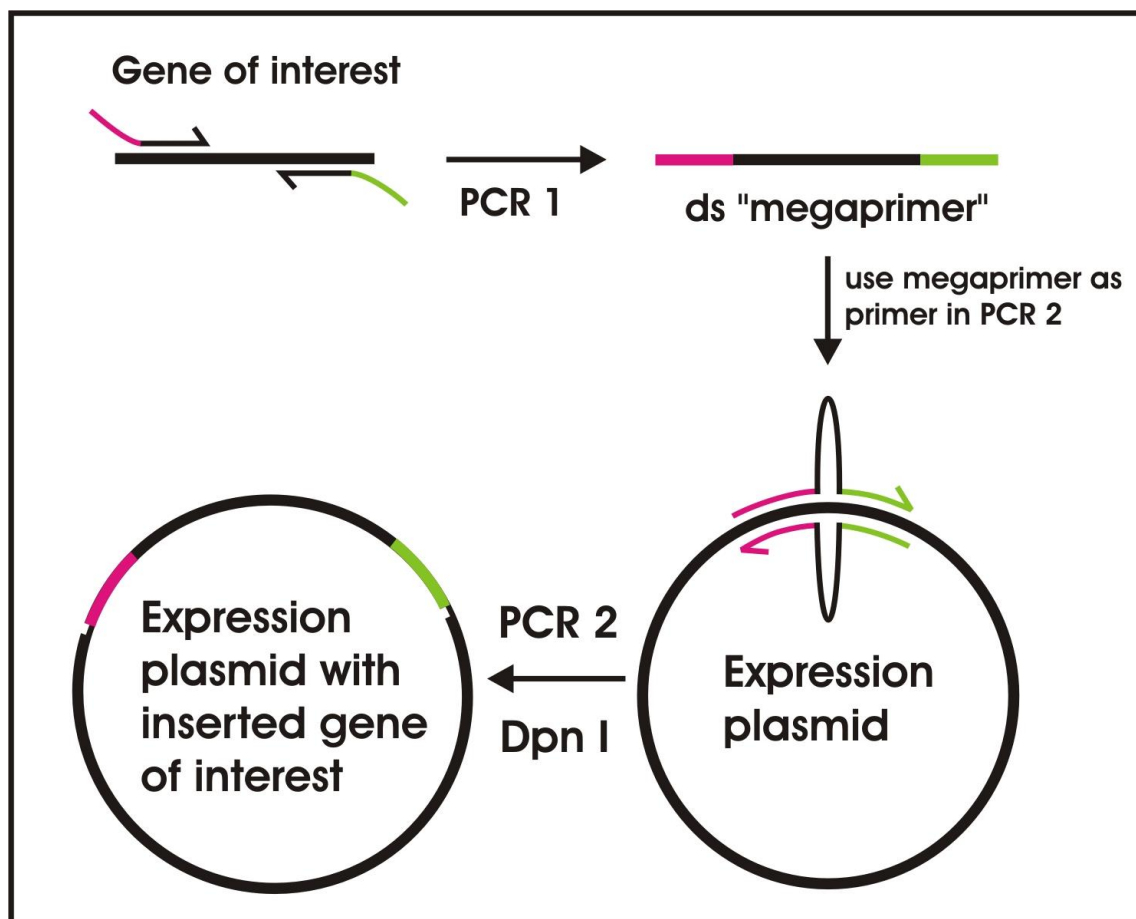


Figure 5) A schematic diagram of integration PCR. Double stranded DNA is represented as a wide single line. The DNA of interest is first amplified in PCR 1, using primers with 5' tails (magenta and green) carrying homology to the site of insertion of an expression plasmid. The PCR product is purified and then used as primers in a second round of PCR (more precisely, a linear PCR - like reaction). During the second PCR, the gene of interest is integrated into the expression plasmid. The circular product contains one nick per DNA strand, however, the two nicks are sufficiently separated from each other to allow strong hydrogen bonding between the single stranded regions. The product can therefore directly be used to transform competent, *E. coli* cells, without ligation.

### In-Fusion™ Cloning

The In-Fusion™ cloning system is available from BD Biosciences. The In-Fusion™ enzyme renders terminal, 15 nt long homology sequences of linear DNA molecules single stranded by strand displacement, and joins complementary single stranded DNA ends (BD In-Fusion PCR Cloning Kit, 2002).

This cloning system can be used for high throughput cloning (BD In-Fusion Universal PCR Cloning System, 2003). Ligase is not needed. Any DNA fragment of interest can be inserted into expression plasmids via In-Fusion™ cloning. Primers for insert amplification need to be designed so that the ends of the PCR products contain 15 bp homology to the ends of the linear plasmids. Insert and linear plasmid are mixed and incubated with the very convenient dry-down In-Fusion mix and incubated for 30 minutes. The mixture can then directly be transformed into Fusion-Blue™ competent *E.*

*coli* cells. An optional purification step prior to transformation increases transformation efficiency. Linear plasmids are available from BD Biosciences. If the available plasmids are not suited for the planned experiment, any vector can be used because the homology regions can be freely chosen. Linearization of plasmids is carried out by restriction enzyme digestion. DNA fragments up to 8 kb in length can be inserted with this protocol. The advantage of this method is that the integration step appears to be more reliable than the ligation step in a classical cloning experiment. Other than that, the usefulness of this method is comparable to classical cloning, because of the dependency on either available linearized vectors or restriction sites for linearization. Furthermore, tedious gel purifications are recommended in the standard protocol.

A schematic representation of how this cloning method works is shown in Figure 6.

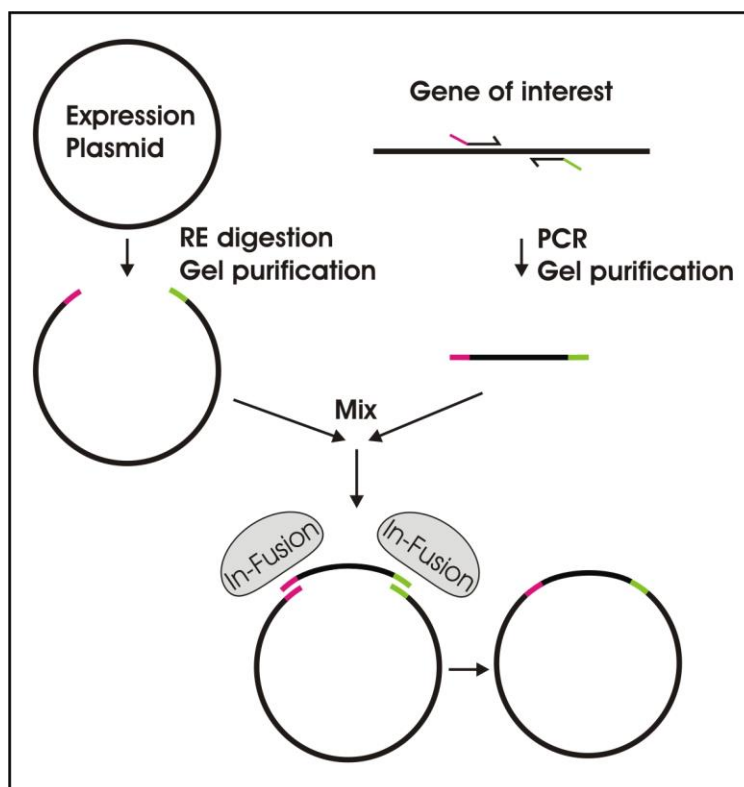


Figure 6) A schematic representation of In-Fusion™ cloning (standard supplier protocol). Double stranded DNA is represented as a wide single line. The plasmid is linearized by restriction enzyme digestion and purified by gel electrophoresis. The insert is produced by PCR, with primers that contain 5' tails carrying homology to the DNA ends of the linearized plasmid (homologies are shown in magenta and green). Purified insert and plasmid are combined, mixed and incubated with the In-Fusion™ dry down mix at 42° C for 30 minutes. The In-Fusion™ enzyme renders the terminal homology regions single stranded by strand displacement and may also have some influence on annealing. After the incubation, 1 ul of the mixture can directly be used to transform chemically competent *E. coli* cells.

### Cloning with TypeIIs restriction endonucleases

Type IIs restriction endonucleases are enzymes that cut DNA outside of their recognition sequence. They recognize an asymmetric sequence. Type IIs restriction enzymes consist of two domains, one is responsible for DNA binding, the other for cleaving. Experiments can be designed so that the recognition sequences of the typeIIs restriction enzyme are removed upon cleavage, by placing the recognition sequences inside the multiple cloning site, in an orientation that leads to digestion 5' of the first recognition site, and 3' of the second recognition site of the leading strand. Because cleavage takes place at a site that is outside of the recognition sequence, the base composition of the overhangs can be freely

chosen, and it is therefore possible to create two different cohesive ends by digestion with a single enzyme.

Type II restriction enzymes are frequently used in combination with other methods. Stratagene, for instance, offers the Seamless<sup>®</sup> cloning kit that combines PCR linearization of plasmids with type II restriction enzyme cloning using Eam 1104 I. The last five cycles of PCR linearizations and amplifications in this system are carried out in the presence of <sup>m5</sup>dCTP in order to protect Eam1104 sites in the vector backbone or insert from digestion. Successful cloning with this strategy has been described (Padgett and Sorge, 1996).

### **TOPO Cloning**

TOPO<sup>®</sup> Cloning takes advantage of the fact that Topoisomerase can cleave as well as join DNA strands. In nature, topoisomerases control the supercoiling of DNA. During RNA transcription and DNA replication, complementary DNA strands need to be separated, resulting in torsional strain. Topoisomerase I catalyzes the relaxation of negative DNA supercoils by creating single stranded DNA breaks and re-ligating them. Type I Topoisomerases, also known as nicking – closing enzymes, are monomeric proteins with a molecular weight of approximately 100 kD. They are found in prokaryotes and eukaryotes. In prokaryotes, they form a phosphotyrosine diester linkage to the 5'-terminal phosphoryl group of linearized DNA after incubation with single stranded circular DNA. Eukaryotic type I topoisomerases, on the other hand, form a similar type of link, however to the 3' end of the DNA. This covalent bond formation between enzyme and DNA has the advantage that the free energy of the cleaved phosphodiester bond is preserved and can be used to reseal the nick without additional energy input. The 67 kD N-terminal fragment of *E. coli* Topoisomerase I folds into 4 domains, forming a hole in the middle, whose surface mainly exposes positively charged residues that are involved in DNA binding.

The Invitrogen TOPO<sup>®</sup> cloning systems use topoisomerase I from Vaccinia virus. According to Shuman, this enzyme consists of 314 amino acids (Shuman, 1994). It recognizes the sequence CCCTT near 3' ends, binds to double stranded DNA and then cleaves a phosphodiester bond of one strand. A covalent bond is formed between the 3'-phosphate of the nicked DNA strand and Tyrosine 274 of the enzyme. The protein can then either religate the strand it broke, or it can ligate another DNA molecule with a compatible 5'-OH tail, creating a recombinant molecule. Vaccinia topoisomerase is very sequence specific.

Because the enzyme only makes single stranded cuts, vectors need to be linearized. The donor strand has to contain the sequence CCCTT at or near the 3' end (maximum distance of 10 bp) and the acceptor strand has to contain a terminal 5'-OH (Shuman, 1994).

Linearized TOPO<sup>®</sup> vectors with covalently bound Topoisomerase I at each 3' end are available from Invitrogen<sup>™</sup>, allowing rapid combination with DNA fragments containing compatible ends. Once insert and plasmid are combined, the covalently bound enzyme is released. Three different systems are available: TOPO TA Cloning<sup>®</sup>, Zero Blunt<sup>®</sup> TOPO<sup>®</sup> Cloning and Directional TOPO<sup>®</sup> Cloning. They differ by the way in which the inserts are prepared. For TOPO TA Cloning<sup>®</sup>, inserts are amplified, or treated, by Taq DNA

polymerase, which has a terminal transferase activity, resulting in single 3' deoxyadenosine overhangs. The inserts are therefore compatible with the TOPO TA Cloning<sup>®</sup> vectors, which have the Topoisomerase I linked to single 3' Thymidine overhangs of the linearized vector.

For Zero Blunt<sup>®</sup> TOPO<sup>®</sup> Cloning, inserts need to have blunt ends, which makes them compatible with the blunt ends of the Zero Blunt<sup>®</sup> TOPO<sup>®</sup> Cloning vectors with Topoisomerase I linked to the 3' Thymidines.

For Directional TOPO<sup>®</sup> Cloning, PCR Products need to have blunt ends, and additionally, they need to contain the sequence CACC at the 5' end. The corresponding linear vectors contain a single stranded GTGG 5' overhang at one end. The overhang invades the double stranded DNA of the insert and anneals to the complementary CACC. The enzyme then ligates the insert in the correct orientation.

The protocol for insertion of DNA fragments is the same for all three systems: First, PCR product and TOPO<sup>®</sup> Cloning vector are combined in a solution. After 5 minutes of incubation at room temperature, the solution is directly transformed into competent *E. coli* cells.

TOPO<sup>®</sup> Cloning has many advantages over classical cloning methods. Primers for amplification of the insert do not need to contain any long non-annealing 5' tails, as is the case when using restriction enzymes.

Linearized, TOPO<sup>®</sup> activated plasmids for many different uses are commercially available. PCR products do not need to be modified or purified prior to ligation. Ligation itself only takes 5 minutes at room temperature.

Unfortunately, there are also some disadvantages. Most importantly, the 5'-(C/T)CCTT-3' sequence flanking the cloning site is fixed, leading to unwanted amino acids in the expressed protein, which can be a major problem if the protein is used for crystallization. Furthermore, the method is non-directional, with the exception of Directional TOPO<sup>®</sup> Cloning, which requires additional unwanted basepairs. With the current systems, unwanted amino acids in the end product can only be avoided if the insert is cloned into TOPO<sup>®</sup> Cloning Expression vectors so that the first inserted codon is the start ATG. Cleavable fusion tags that simplify purification or enhance solubility could therefore not be used in this context, unless tag (if short enough) and protease recognition site are added to the inserts via non-annealing 5' tails, resulting in very long primers and therefore increased mutation probability.

### **Homologous recombination (ET cloning / RecA-dependent cloning)**

ET cloning is a cloning system that is based on homologous recombination, using the enzymes recE and recT (Muyrers et al., 1999). PCR products that are flanked by 50 bp of homology to the desired location of integration can be directionally inserted into circular DNA molecules. The method has been described to work well for the modification of bacterial artificial chromosomes (BACs) in their host strains.

This type of recombination works only in *sbcA E. coli* strains, because these strains express RecE and RecT (Zhang et al., 1998). Homology regions up to 60 bp in length have been examined, and within this range, recombination efficiency increased when longer homology regions were used. The method worked with a minimum homology length of 27 bp, however, efficiency was very low in this case.



ET cloning is an in-vivo process. Inserts containing sufficiently long terminal regions that are homologous to the sites of insertion of the expression plasmid, and the expression plasmid, are co-transformed into *E. coli* cells that express either the RecE and RecT proteins from the Rac Prophage or the Red $\alpha$  and Red $\beta$  proteins from lambda phage (Zhang et al., 2002). Both pairs of enzymes are involved in initiating the repair of double strand breaks in DNA. RecE and Red $\alpha$  have 5'-3' exonuclease activity, resulting in the creation of a single stranded 3' overhang. RecT and Red $\beta$  bind single stranded DNA. The single stranded overhang / enzyme complex probably invades the double stranded DNA, leading to recombination upon DNA-replication. ET cloning is useful for the insertion of point mutations as well as for the insertion of DNA fragments up to 80 kb without the need for gel purification. Furthermore, deletions of 1 bp – 70 bp are possible. ET cloning is seamless because homology regions can be freely chosen. Cloning efficacy has been reported to be much higher than in with RecA dependent cloning methods. According to Zhang et al., mutations do not occur because the method uses endogenous *E. coli* mechanisms for cloning.

One drawback appears to be the extremely long primer tails, which are likely to render successful PCR reactions difficult. Furthermore, homologous recombination is an event that occurs only rarely (Muyrers et al., 2001), and cloning experiments therefore often have to be carried out in two rounds. First, the gene of interest is integrated at the desired site along with a selectable marker that allows identification of colonies containing the desired product. Next, the selectable marker is removed.

RecA-dependent cloning is another strategy of homologous recombination. Recombination can be achieved between two circular molecules (Muyrers et al., 2001). The recombination takes place through the homology regions. Like ET cloning, RecA cloning also requires tedious selection procedures.

### Site-specific recombination

#### Gateway<sup>®</sup> Technology

In site-specific recombination, specific DNA sequences are cleaved and rejoined (Landy, 1989). DNA synthesis is not involved in this process. In  $\lambda$  recombination, four proteins are responsible for the integration and excision reactions. Four recombination sites are involved. They are called “att” sites. Strand exchange takes place when DNA is cut and resealed by the integrase protein Int, a basic, 40 kDa protein with topoisomerase I activity. Other proteins may also be involved in the reaction.

Gateway is a very efficient and modular method for inserting DNA fragments into a variety of expression vectors. It uses lambda phage-based site-specific recombination. DNA fragments need to be flanked by recombination sites for this purpose. The gene of interest is first cloned into an entry vector by directional TOPO<sup>®</sup> Cloning, PCR Cloning with Gateway<sup>®</sup> Technology, or Restriction Cloning. The entry clone is then mixed with any Gateway<sup>®</sup> expression vector and the Gateway LR Clonase<sup>™</sup> enzyme mix. The LR Clonase<sup>™</sup> enzyme mix catalyzes the in vitro recombination between the entry clone, with the gene of interest flanked by attL sites, and the expression (destination) plasmid, containing attR sites. This site-specific recombination event results in an expression clone

and in a side product. The mixture is then transformed into competent *E. coli* cells. The cells are plated out on selective medium.

The strength of this system is that any gene of interest can easily be directionally inserted in the correct reading frame into a wide variety of expression vectors. Furthermore, only the entry vector needs to be sequenced. Gateway<sup>®</sup> Technology is probably the most powerful method currently available for high throughput expression screening.

Unfortunately, the system is not ideal for the multiparallel production of proteins for x-ray crystallographic applications, because the fixed recombination sites introduce the eight unwanted amino acids TSLYKKAG (translation of attB1 site) to the N-terminus of the final translation product. The amino acid directly following the eight fixed residues is also not freely choosable, because the first base in its DNA sequence has to be thymine. If a protease recognition site is positioned towards the N-terminus of the protein of interest, in such a way that it is transferred from the entry vector to the destination vectors together with the gene of interest, then the system could be used in structural biology for the screening of different tags and fusion proteins. Constructs of different length, however, have to be constructed by conventional methods.

#### The Cre/loxP system

The Cre/loxP recombination system has been derived from the P1 bacteriophage (Kühn and Torres, 2002). Cre recombinase is a member of the integrase superfamily of site-specific recombinases. The 38 kDa enzyme recognizes the locus of crossover in P1 bacteriophage (loxP site). LoxP sequences consist of two 13 bp long inverted repeats that flank an 8 bp nonpalindromic sequence, leading to directionality. The loxP sequence is shown in Table 1. Cre is most active at 37° C and is therefore well suited for cloning experiments in mammalian cells. According to the current model, four Cre molecules bind to two loxP sites and form a four-way Holliday-junction intermediate. The parental DNA strand is cleaved by a tyrosine residue of the enzyme, resulting in a covalent 3'-phosphotyrosine intermediate. The free 5' OH groups attack the phosphotyrosines of the opposing strand, leading to the Holliday-junction, which is resolved by tyrosine cleavage, linkage and strand exchange. The Cre/loxP system has been widely used for a long time, especially for experiments involving the genome of mice or embryonic stem cells.

Table 1) The loxP sequence (Kühn and Torres, 2002). The nonpalindromic spacer is shown in bold and underlined.

<p>5'-ATAACTTCGTATAG<b><u>CATACATT</u></b>TATACGAAGTTAT-3'  3'-TATTGAAGCATAT<b><u>CGTATGTA</u></b>ATATGCTTCAATA-5'</p>
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#### Enzyme-free cloning

Sticky end PCR has been described above (3.1.4.). Enzyme-free cloning (Tillett and Neilan, 1999) works the same way, except that not only the inserts, but also the plasmids are produced by denaturation and reannealing of two PCR products that contain the same core, but a slight offset at the ends. An offset of 12 – 18 basepairs results in sufficiently strong hydrogen bonding between cohesive ends of insert and plasmid to allow direct transformation without ligation. The advantage of this method is that it is independent of

enzymatic *in-vitro* reactions after PCR. The disadvantage is that each experiment requires eight primers and four PCR reactions. A schematic representation of enzyme-free cloning is shown in Figure 7.

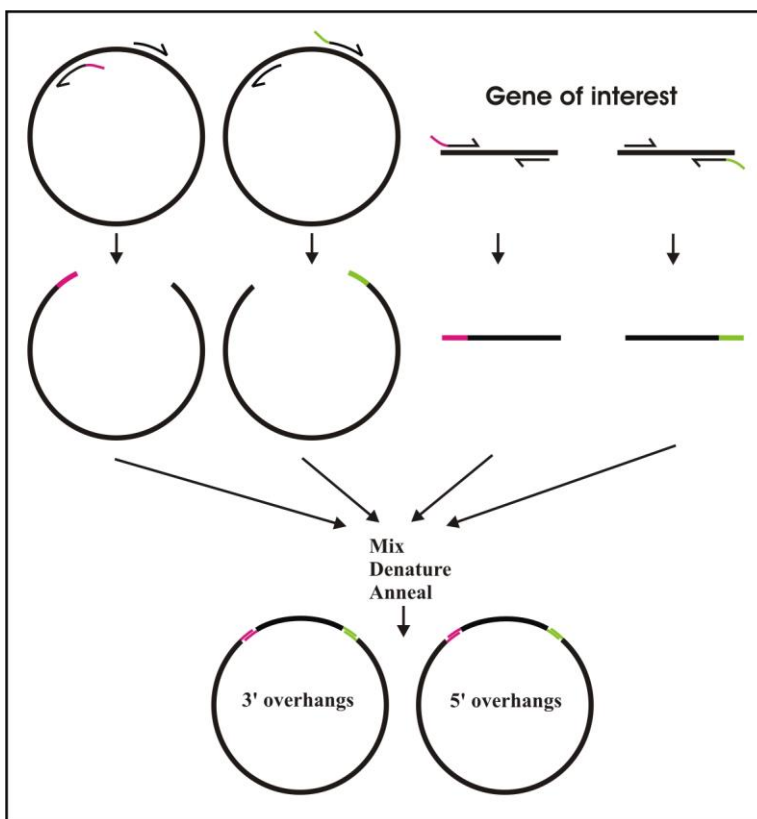


Figure 7) A schematic diagram of enzyme-free cloning. Double stranded DNA is represented as a wide single line. Both, plasmid and insert, are PCR amplified in two reactions each. Non-annealing 5' primer tails are added to different primer primers in the two PCR reactions, leading to PCR products with the same core, but a slight offset at the ends. Heat denaturation and reannealing of a mixture of all plasmid and insert PCR products leads to circular plasmid. Inserts are attached to the plasmids via hydrogen bonding at compatible homology regions, resulting from the overhang offset in the primers. If the compatible single strands are sufficiently long, the product can be directly transformed without need for ligation.

### Ligation independent cloning (LIC)

Ligation independent cloning is a method that generates recombinants between PCR products and PCR amplified plasmids without the use of restriction enzymes, DNA ligase or alkaline phosphatase (Aslanidis and de Jong, 1990). 12 nucleotides, lacking dCMP, are added to the 5' end of each insert amplification primer, leading to PCR products with 12 nt long ends lacking dGMP in their 3' ends. In the presence of dGTP, 5' extending single strands of defined length can then be created by taking advantage of the 3' – 5' exonuclease activity of T4 DNA polymerase. Complementary 5' extending single strands in the plasmids can be generated in a similar way, by adding 12 nt long 5' ends to vector amplification primers that are complementary to the tails used for insert amplification. 3' – 5' exonuclease reaction is in this case carried out in the presence of dCTP. The created cohesive ends lead to directional cloning, and only vectors with inserts can circularize, so that background levels (transformation of plasmids without insert) should be very low. No ligation is necessary, because a 12 nucleotide long annealing region is sufficiently stabilized by hydrogen bonding to allow transformation of circular product, which is then repaired in the cell to form an intact plasmid that can replicate.

The advantage of this method is that the ligation step, which is often the failing step in classical cloning experiments, can be avoided. The method is however too complicated to be used for high throughput cloning, because of the very selective reaction conditions

(presence of only dGTP or dCTP). Furthermore, the site of insertion needs to be deficient of specific nucleotides, so that seamless cloning is not possible except in special situations.

### **PCR-induced subcloning**

PCR-induced subcloning allows the directional subcloning of PCR fragments in a single day (Shuldiner et al., 1990). DNA ligase is not used in the protocol. First, the gene of interest is amplified with primers containing 24 nucleotides long non-annealing 5' tails that are complementary to the ends of the site of insertion of a linearized plasmid. Excess primers are removed by filtration through a Millipore Ultrafree MC unit. 50 ng – 100 ng of PCR product are then placed into two separate tubes containing 50 ng of linearized plasmid, as well as PCR buffer, dNTPs, and Taq polymerase. Furthermore, reaction A obtains two additional primers, and reaction B obtains two different additional primers. In the first PCR cycle, the amplified DNA fragment anneals to the linearized plasmid via the 24 complementary nucleotides, and elongation leads to full length, double stranded, linear plasmid plus insert. In the remaining PCR cycles, the additional primers amplify the linear plasmid-insert template. Following the PCR, 10 ul of PCR A and 10 ul of PCR B are mixed. The double stranded DNA is then denatured in 0.2 M NaOH for 5 min at room temperature. Next, the mixture is neutralized by addition of 400 ul of boiling 50 mM Tris, pH 6.6. During three to six hours of incubation at 60° C, the complementary strands reanneal. Two of the possible products, one annealing at the complementary insert regions and the other annealing at the complementary plasmid regions, have long, complementary single stranded overhangs, leading to circularization.

The mixture is then concentrated using a Millipore Ultrafree MC unit. The circular products can be directly used to transform competent *E. coli* cells because the sticky ends are sufficiently long to allow direct transformation without ligation. A schematic representation of PCR-induced subcloning is shown in Figure 8.

This original protocol seems quite complicated, many of the steps could probably be simplified. For instance, the NaOH denaturation step could most likely be replaced by a heat denaturation step. Addition of NaCl to 100 mM, as sometimes used in sticky-end PCR, may help in the denaturing – renaturing process. Excess primers could be removed by simple spin column purification.

The advantages of this method are that ligation is not necessary and that the protocol can be carried out in a single day. The protocol involves too many steps in order to be useful for medium to high throughput cloning. The large number of primers that are needed further complicates the workflow.

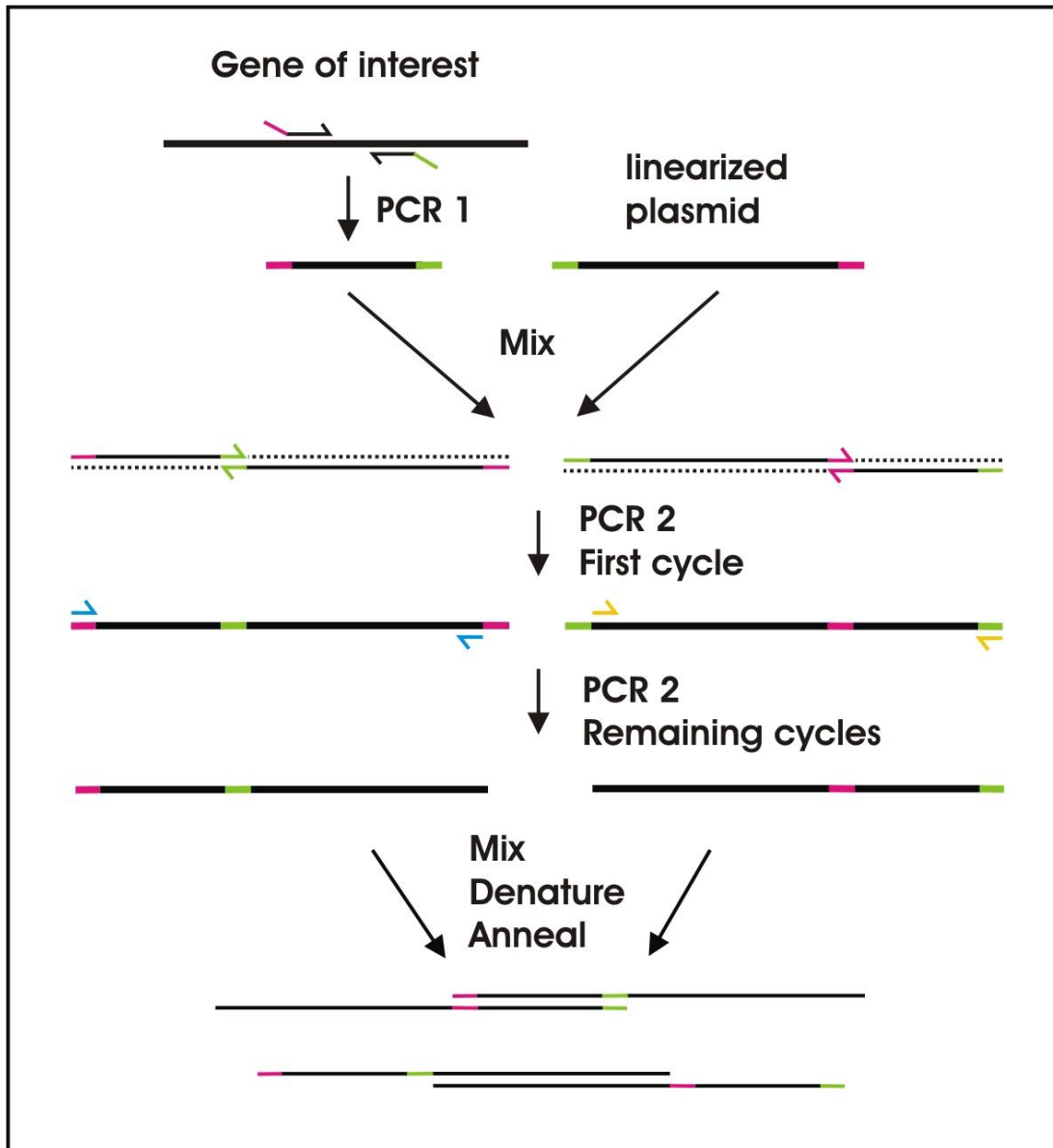


Figure 8) A schematic representation of PCR-induced subcloning. Double stranded DNA is represented as a wide single line. Arrows indicate the direction of primer extension. First, the gene of interest is amplified by PCR. 24 nt long sequences that are complementary to the ends of a linearized plasmid are introduced via 5' non-annealing primer tails. Excess primers are then removed. Plasmid and insert are mixed in two separate tubes. Two different sets of primers are added to each reaction (shown in blue and yellow). A second round of PCR is carried out. In the first cycle of PCR 2, plasmid and insert anneal to each other via the terminal 24 bp complementary regions. Insert and plasmid 3' ends serve as primers, leading to two possible double stranded linear products, each containing both insert and plasmid. In the remaining cycles of PCR 2, the newly added primers (blue and yellow) amplify the linear products of the first cycle. The offset in primer annealing regions leads to the deletion of one of the terminal 24 bp regions found in each template DNA. Upon mixing, denaturing and annealing, several different products can form, two of which each contain long single stranded, complementary overhangs, leading to circularization. The circular product can be used to transform competent *E. coli* cells.

### Universal restriction site-free cloning with chimeric primers

Rare-earth metal ions can cleave the phosphodiester bond between a ribonucleotide and a deoxyribonucleotide. Universal restriction site-free cloning takes advantage of this site-specific cleavage (Guo et al., 2001). Insert and plasmid are amplified by PCR. Primers are designed so that the ends of the insert PCR product are complementary to the ends of the vector PCR product. Each primer contains a single ribonucleotide, introducing a site that can be specifically cleaved by rare-earth metal ions, i.e. lanthanum chloride ( $\text{LaCl}_3$ ), into the PCR products. Incubation of PCR products at  $50^\circ\text{C}$ , in the presence of  $\text{LaCl}_3$  ( $V_f = 10\text{ mM}$ ) at pH 9.5 leads to hydrolyzation of the bond, resulting in the creation of 3' overhangs that can be used for directional ligation after a phosphorylation step. Figure 9 shows a schematic representation of restriction site-free cloning with chimeric primers.

This method looks far too complicated for routine use. The need for chimeric primers alone already complicates the protocol, and probably increases the cost of each experiment. Purification steps, such as gel electrophoresis of PCR products and precipitation of the rare-earth metal ion treated PCR products, render this protocol work intensive and tedious. Only DNA Polymerases that can use RNA as a template can be used for this method.

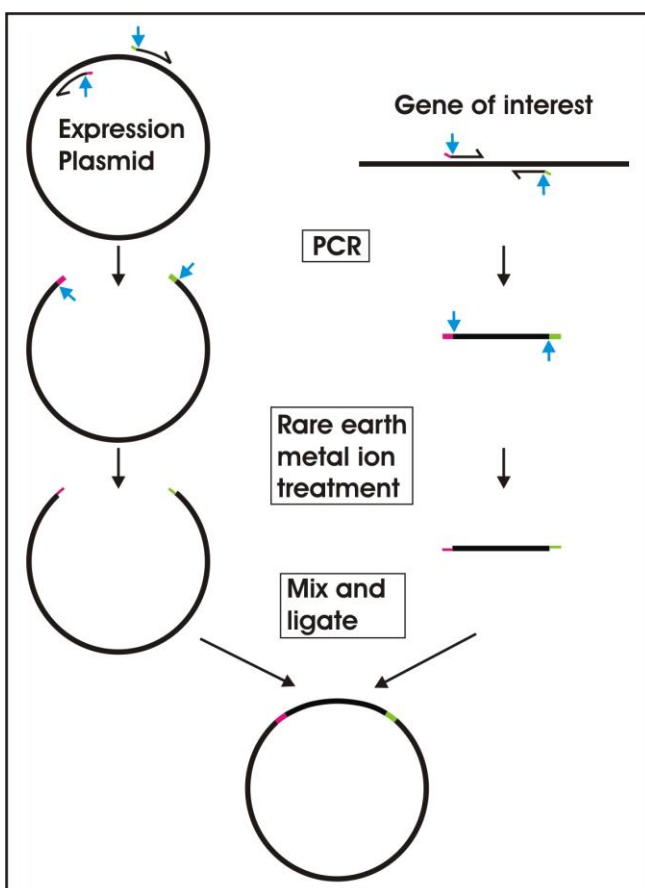


Figure 9) A schematic representation of restriction site-free cloning with chimeric primers. Double stranded DNA is represented as a wide single line. Plasmid and insert are both amplified by PCR. Primers for plasmid linearization contain 5' ends with homology to the 5' ends of the primers used for insert amplification. The two different homologies are shown in magenta and green. Primers contain a ribonucleotide between the annealing part and the homology part (indicated by the blue arrows). Upon incubation with lanthanum chloride, the phosphodiester bond between a ribonucleotide and a deoxyribonucleotide are cleaved, leading to the creation of single stranded overhangs that can be used for sticky end ligation after a phosphorylation step.

### Rapid gene cloning using terminator primers and modular vectors

This method allows the assembly of several genes by combining PCR products. Complementary sticky ends of sufficient length for ligation-independent cloning (12 nt or longer) are produced by using terminator primers (Donahue et al., 2002). These primers consist mainly of DNA, but include either up to three ribonucleotides following each other, or a single 2'-O-methyl ribonucleotide. The purpose of the ribonucleotides, or of the 2'-O-methyl ribonucleotide, is to terminate DNA polymerase at the position of the non-DNA components, leading to single stranded overhangs that can be used for annealing to DNA fragments with complementary sticky ends. Pfu exo(-) DNA polymerase and Taq DNA polymerase terminate prior to copying a 2'-O-methyl ribonucleotide, but only after copying some of the ribonucleotides. Cloning efficiency is therefore considerably higher when using primers with a single 2'-O-methyl ribonucleotide, compared to primers containing a single ribonucleotide. With this method, it is possible to join more than two DNA fragments at the same time.

For standard insertions of genes of interest into expression regions of plasmids, the need for chimeric primers may be a drawback because it complicates the primer ordering procedure and is likely to increase costs. A schematic representation of rapid gene cloning using terminator primers and modular vectors is shown in Figure 10.

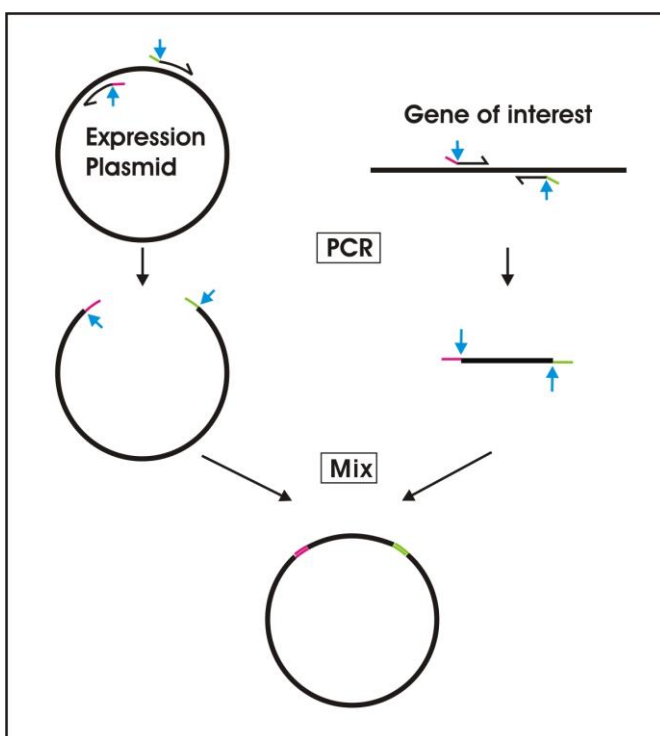


Figure 10) A schematic representation of rapid gene cloning using terminator primers and modular vectors. Double stranded DNA is represented as a wide single line. Plasmid and insert are both amplified by PCR. Primers for plasmid linearization contain 5' ends with homology to the 5' ends of the primers used for insert amplification. The two different homologies are shown in magenta and green. Primers contain one to three ribonucleotides or a 2'-O-methyl ribonucleotide between the annealing part and the homology part (indicated by the blue arrows). PCR is carried out with a DNA polymerase that cannot use RNA as a template, leading to termination of polymerization at the position of the ribonucleotide(s), leading to compatible single stranded overhangs. If these overhangs are sufficiently long, the product can directly be used to transform competent *E. coli* cells, without ligation.

## Mutagenesis

### QuikChange<sup>®</sup> site directed mutagenesis (Stratagene)

QuikChange<sup>®</sup> site directed mutagenesis is probably the most popular method for the generation of short mutations, deletions or insertions in plasmids. Two primers are designed to anneal to different strands of a plasmid, towards both sides (5' and 3') of the region that is to be mutated. The primers contain the mutation somewhere near their center, leading to a region in the primers that cannot fully anneal to the template. The reaction is cycled in a similar way as for a regular PCR, except that elongation times are kept very long in order to allow copying of the full plasmid, and the elongation temperature is lowered to 68°C. The protocol takes advantage of the fact that Pfu DNA Polymerase does not strand-displace at 68°C. Elongation therefore stops when the Polymerase has traveled around the plasmid and reached the 5' end of the mutagenesis oligonucleotide that was used for priming. This is not a true polymerase chain reaction. Only annealing to the parental template leads to the correct product, therefore, this is a linear reaction, with the advantage that chances of introducing mutations are lower than in an amplification reaction. Once cycling is complete, parental template is digested by incubation with Dpn I, and the product is used to transform competent *E. coli* cells for later plasmid purification and sequencing. A schematic representation of the method is shown in Figure 11.

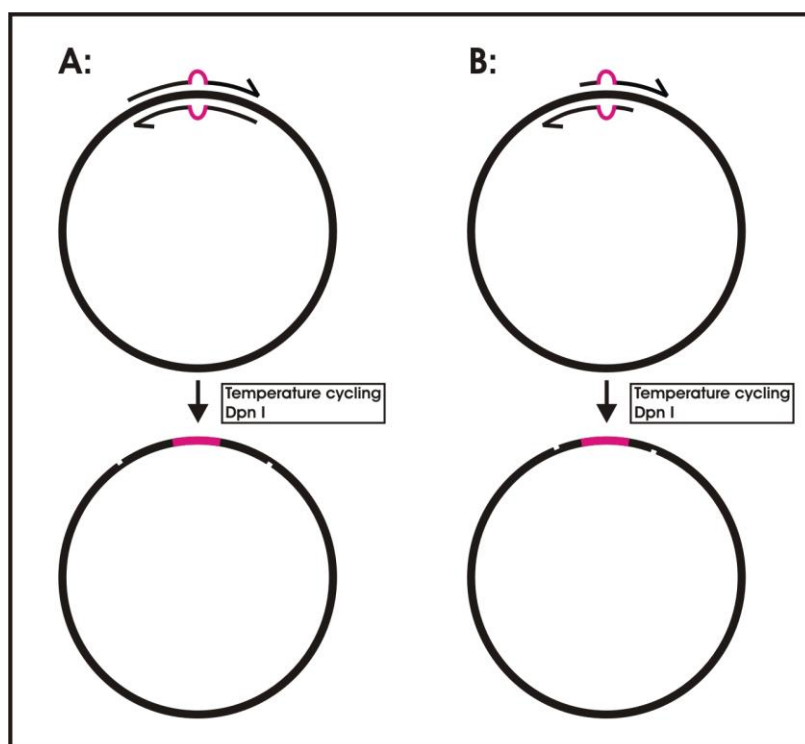


Figure 11) A schematic representation of QuikChange<sup>®</sup> site directed mutagenesis. Double stranded DNA is represented as a wide single line. A) The standard protocol. Two primers, containing the mutation anneal to different strands of the plasmid and run in opposite directions. The full plasmid is copied. The offset in position of the 5' ends of the primers leads to a circular product with single stranded nicks that are sufficiently far apart from each other to allow transformation of unligated product. B) The method often works better if primers are only partially complementary to each other (Zheng et al., 2004)

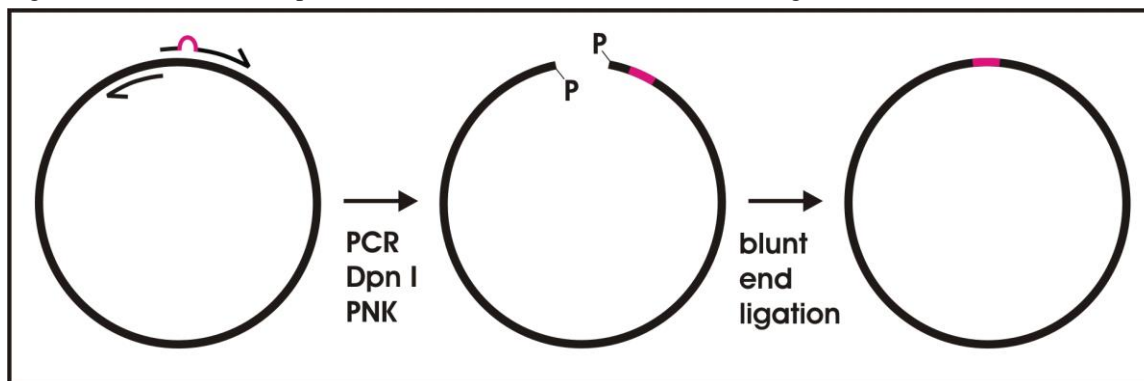


### ExSite Site directed mutagenesis (Stratagene)

For ExSite site directed mutagenesis, two primers that run in opposite directions on different strands of a plasmid are designed. One of the plasmids contains the desired mutation at or near the 5' end. During PCR, the full plasmid is amplified, resulting in a linear, double stranded DNA product that contains the mutation or insertion towards one of the ends. The linear DNA product is then phosphorylated by treatment with polynucleotide kinase in the presence of ATP. Circularity is restored by blunt end ligation. The advantage of this method over QuikChange site directed mutagenesis is that the primers do not have to contain long complementary regions that could interfere with priming. The disadvantage is that a blunt end ligation step is involved in the protocol.

It is possible to produce sticky ends via restriction enzyme cleavage sites near the 5' ends of the primers, or by inserting homology regions at the 5' ends of the primers and chewing off one strand with appropriate DNA modification enzymes. Efficiency may be improved in this way, however, the protocol is rendered more complicated due to additional enzymatic steps. Unlike QuikChange site directed mutagenesis, ExSite site directed mutagenesis is a true PCR reaction, leading to a higher risk for the introduction of unwanted mutations in the vector backbone. A schematic diagram illustrating the main steps of the protocol is shown in Figure 12.

Figure 12) A schematic representation of ExSite site site directed mutagenesis. Double stranded DNA is



represented as a wide single line. Two primers are designed to anneal to different strands of a plasmid. The primers run in opposite directions. One of them carries the mutation at or near the 5' end. The full plasmid is amplified by PCR, leading to a linear product that contains the inserted mutation at or near one end. Template plasmid is removed by Dpn I digestion. The PCR product is then phosphorylated. Plasmid recyclization is achieved by blunt end ligation.

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