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'Restriction-PCR' - a Superior Replacement for Restriction Endonucleases in DNA Cloning Applications

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Polymerase chain reaction (PCR) is well established as an indispensable tool of molecular biology; and yet a limitation for cloning applications continues to be that products often require subsequent restriction digests, blunt-end ligation, or the use of special linear vectors. Here a rapid, PCR-based system is described for the simple, restriction enzyme-free generation of synthetic, 'restriction-like' DNA fragments with staggered ends. Any 3'- or 5'-protruding terminus, but also non-palindromic overhangs with an unrestricted single strand length are specifically created. With longer overhangs, "Restriction-PCR" does not even require a ligation step prior to transformation. Thereby the technique presents a powerful tool e.g. for a successive, authentic reconstitution of sub-fragments of long genes with no need to manipulate the sequence or to introduce restriction sites. Since restriction enzyme-free and thereby devoid the limitations of partial DNA digests, "Restriction-PCR" allows a straight one-step generation and cloning of difficult DNA fragments that internally carry additional sites for those endonucleases involved in the cloning. Small site-specific sequence insertions or deletions can be precisely engineered into genes of interest. With these properties "Restriction-PCR" has the potential to add significant speed and versatility to a wide variety of DNA cloning applications.

Keywords: Cloning, Ligase-free, PCR, Partial digest, Restriction endonuclease.

Introduction

Besides the use in a simple target amplification, PCR technology is today being widely used for cloning, or for oligonucleotide guided DNA mutagenesis. Often in these latter applications the amplified DNA material requires a precise adaptation of its termini. This step involves the introduction of new and unique restriction sites via the PCR primers in order to mobilize the PCR fragment of interest, and necessitates a subsequent digest with the respective restriction nucleases. Alternatively, the terminal nucleotide transferase activity of the non-proofreading polymerases (Clark, 1988; Newton and Graham, 1994) can be utilized, however, at the price of fidelity. The created 3' A-overhang facilitates a direct ligation of the PCR product into a special, linearized T-overhang plasmid vector (Mezei and Storts, 1994). These are major limitations of these techniques. Only these restriction sites are suitable sites for the termini, which are not found within the fragment of interest. Often the desired vector for cloning and expression does not possess a convenient restriction site for insertion, or it is in an undesirable place. Cloning of a special insert of interest, particularly where only a limited sequence information is available, becomes quite a time consuming sequence of trial and error. By introducing "Restriction-PCR", we present a versatile alternative technique, which utilizes a dual proofreading PCR reaction with two specifically designed primer pairs and subsequent in vitro heteroduplex formation of the products.

Materials and Methods

Plasmids pNL-BX is a pNL4-3 derivative lacking most of its flank and carrying several point mutations in gag, pol and env; pGFPuv was purchased from Clontech AG (Basel, CH). Bacteria: HB101/I were grown in LB medium or on LB plates in the presence of 100 µg/mL Ampicillin; competent bacteria were prepared according to standard $CaCl_2$-protocols; a 1 hr recovery phase at 37°C in LB-medium without antibiotic was allowed prior to plating. Restriction endonucleases Bam HI, Xma LI, Cla I came from Life Technologies AG, Basel (CH); enzymes Kpn I, Eco RI, ligase, Pfu-polymerase, and calf alkaline phosphatase were purchased from Promega/Catalys AG (Wallisellen, CH). Thermocyclers PE2400 and PCR-reagents were from Perkin Elmer (Foster City, USA). All fine chemicals were from Merck (Darmstadt, GER), bacterial media came from Difco (Detroit, USA). PCR-purification columns and plasmid-miniprep kit were

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obtained from Qiagen (Hilden, GER).

All purified oligonucleotides came from Life Technologies AG (Basel, CH). The primer sequences used for the Kpn/Eco "Restriction-PCR" were: Kpn-o.dir: gtaccagttagagaaagaac; Kpn-i.dir: cagttagagaaagaac; Eco-o.RC: aattctgatgcacaaaatagagtg; Eco-i.RC: ctgatgcacaaaatagagtg. For GFP enzyme-free cloning with 20bp overhang: GFP1.dir: tagcagaccattatcaacaa; GFP2.dir: aacaaaatactccaattggc; GFP3.RC: cttactgtcatgccatccgt; GFP4.RC: tccgtaagatgcttttctgt; GFP5.dir: tggcatgacagtaagagaat; GFP6.dir: agaattatgcagtgctgcca; GFP7.RC: gataatggtctgctagttga; GFP8.RC: gttgaacggatccatcttca. Standard-PCR conditions were: 5 min 94°C, then 30 cycles under these conditions: 15 sec. 94°C; 15 sec. 52°C; 150 sec. 72°C.; then 5 min. at 72°C. DNA-reannealing prior to PCR was typically performed in 1x PCR-buffer, and was often unsuccessful when done in pure water! The DNA mixture was denatured by heating the tube to 95°C in a vessel with ca. 20 µL of H_2O and then slowly cooling it to 70°C over a period of ten minutes.

Results

The principle of this procedure is based on a statistical re-annealing of thermally denatured DNA strands prior to the cloning steps. Two primer pairs, each shifted by the respective bases that are typical for DNA ends after restriction cleavage (exemplified for a Kpn I site in the sketch of Figure 1a), are used to generate two distinct PCR fragments, which differ solely by the length of their termini. After thermal denaturation in the presence of salt, a stoichiometric mixture of both products is slowly cooled to permit random re-annealing of complementary strands. As depicted in Figure 1, one quarter of the annealed material will possess the correct, staggered termini and will be suitable for immediate ligation into a receptive, e.g. predigested, plasmid vector (Figure 1a: step "->cloning"). As in most PCR-cloning-applications, the absolute DNA amounts are not a limiting parameter and it is not critical that only a quarter of the product is suitable for ligation. In addition, the staggered nature of the ligateable ends gives the product of a "Restriction-PCR" reaction a significant advantage. Previously, the successful production of stable GC-overhangs (Liu, 1996), or of DNA sequence mutations (Ailenberg and Silverman, 1996), have been shown using a related procedure.

Restriction digests become unnecessary. In the first series of experiments we demonstrated that both 5' and 3' protruding restriction-ends can be generated with high fidelity. Fragments carrying "synthetic" KpnI- and EcoRI-'cut' termini were produced by the following "Restriction-PCR" protocol: the KpnI primer set "Kpn-out" and "Kpn-in" was designed to match the KpnI site at position 3826 of the HIV-1 plasmid pNL-NF, a shortened version of pNL4-3 (Fig. 2a: "insert"). The reverse EcoRI primers "Eco-out" and "Eco-in" would generate a new, second EcoRI site at position 6390 of the pNL-plasmid (Figure 2a). The resulting pair of PCR-fragments, extending from 'Kpn-out' to 'Eco-out', and from 'Kpn-in' to 'Eco-in', respectively, had a length of about 2.5 kbp each. Relative amounts of the products were determined from an ethidium bromide stained agarose gel. Equal amounts of both reactions were mixed without further purification, heat-denatured at 95°C in 1x PCR-buffer and re-annealed by

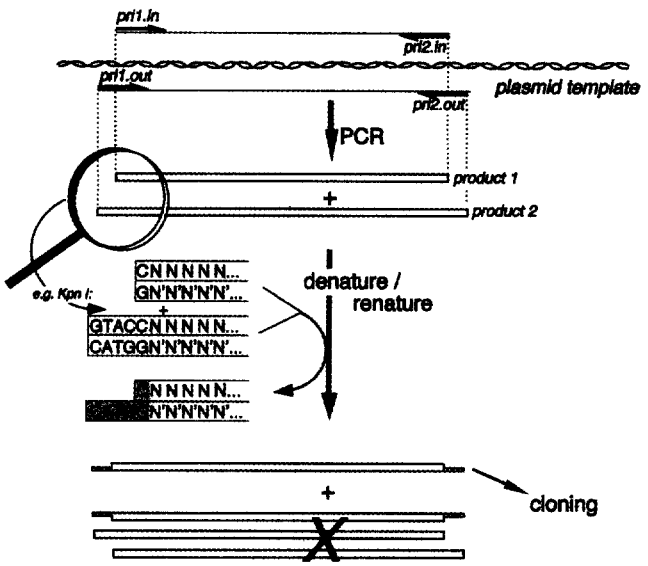

Fig. 1. Principle and use of "Restriction-PCR" to replace 'partial DNA-digests'. The parallel PCR-reactions: "pri1in/pri2in" and "pr1out/pri2out", generate products 1 and 2 with a reconstituted Kpn I restriction site at their left terminus. Mixing, denaturing and renaturation reconstitute a DNA double strand in four possible ways, of which the top one forms a perfect, open Kpn I site (shown on the left as nucleotide sequence), suitable for direct cloning.

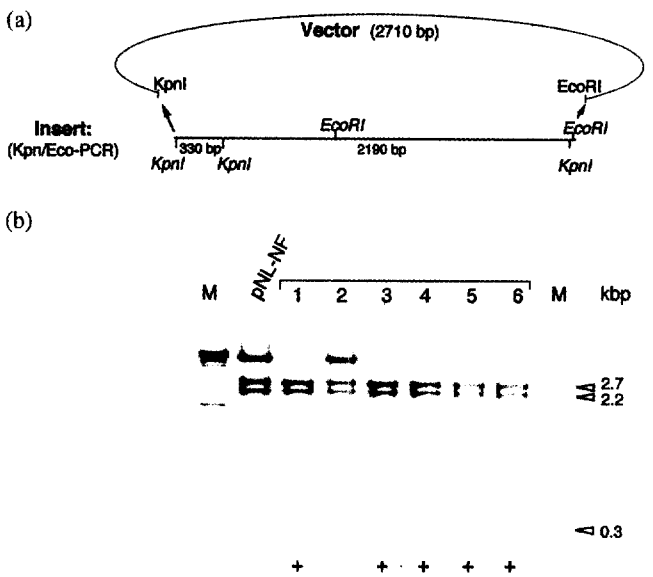

Fig. 2. Agarose gel analysis after Kpn I digest. The "Restriction-PCR"-cloned Kpn/Eco fragment of 2.5 kbp, containing three internal Kpn- or Eco-sites, was cloned into a 2.7 kbp vector (drawing in (a)). Fragment sizes of 0.3, 2.2, and 2.7 kbp indicate faithful cloning in five out of six colonies ("+" in (b)); lane 'pNL-NF' contains the Kpn I digested PCR-template, and lane 'M' the 1 kbp DNA size ladder.

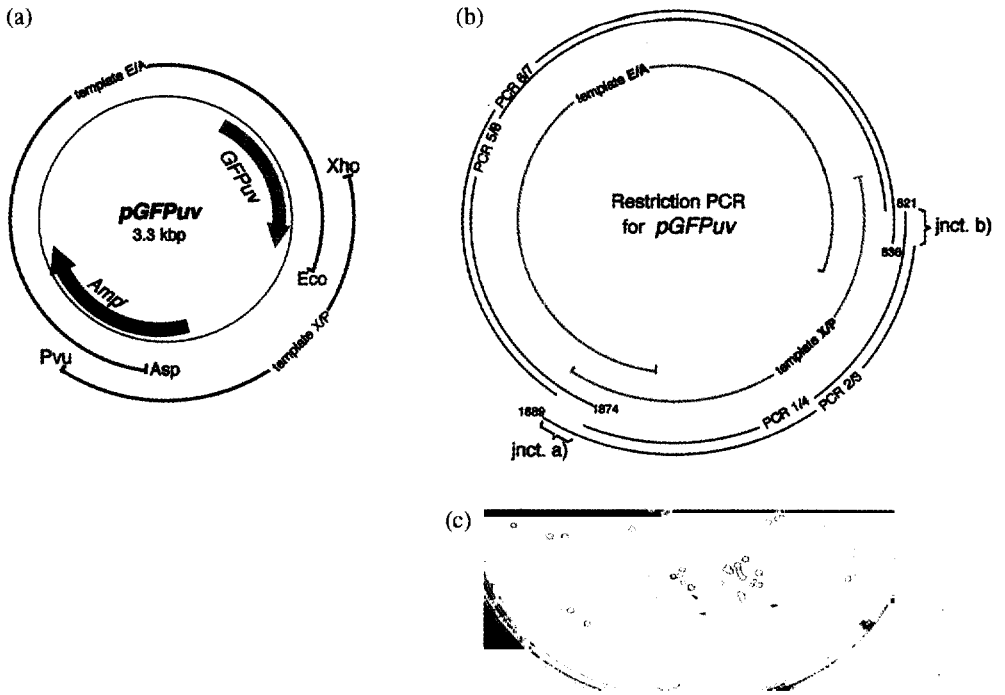

Fig. 3. (a) Scheme for sequence-guided cloning of DNA fragments to reconstitute a functional GFP-plasmid (inner circle). Two segments (templates A/E and X/P) serve as background-free templates for a "Restriction-PCR" reaction (bold lines). (b) Primer pairs (1/4+2/3) and (5/8+6/7) generate the indicated two half-reaction for reconstitution of the pGFPuv plasmid. Numbers indicate the respective nucleotide position on the plasmid. The complementing 15 bp overhangs form junctions 'jnct.a)' and 'jnct.b)'. Reconstituted GFP-competent bacterial colonies are verified by UV-fluorescence (figure c, contrast enhanced image). Non-fluorescent colonies are indicated by arrows ('neg').

cooling slowly to below 72°C over a period of ten minutes. The Kpn/Eco-compatible product was directly ligated into the prepared purified Kpn I/Eco RI vector fragment of pGFPuv (Promega) (Fig. 2a: "vector"), where it replaced the GFP-gene. After bacterial transformation of the ligation mix, without prior removal of the primers or PCR-template DNA, six colonies were picked at random from the plate of transformed bacteria, and the contained plasmids were analyzed. A KpnI restriction digest revealed that all three sites were present and the generation of the expected fragment lengths of 2.2 and 0.3 kbp, in addition to the 2.7 kbp vector fragment (Figure 2b, lanes '+'), demonstrated a faithful insert-ligation. These plasmids also carried the two expected EcoRI sites (not shown). A control reaction, consisting of the identical DNA but rapidly chilled in ice water prior to ligation, produced only few colonies. These exclusively contained the pNL-NF plasmid, which had served as a PCR template for the Kpn/Eco insert-PCR (similar to lane '2' in Figure 2b). They lacked the new, PCR-based insert (not shown). The successful cloning by "Restriction-PCR" of complex inserts, such as the model insert used in this study, which possessed three KpnI sites and two EcoRI sites, demonstrates the power of the method. Already the involved number of five sites would have made conventional cloning by 'partial digestion' impossible. Similarly, "Restriction-PCR" offers an easy way to avoid the limitations through enzymatic 'star' activities that occur in the suboptimal buffer conditions which are often used for simultaneous double digests with two endonucleases etc.. Moreover, this technique renders any multiple-step restriction digests, or the necessity to remove enzymes prior to ligation, superfluous. We observed that "Restriction-PCR" is suitable with similar precision for a variety of 'sites' with their different four-base-overhangs and base compositions (besides KpnI, BamHI and XmaI were tested, not shown) They also work with pure A/T containing termini (Eco RI), or even two-base overhangs, as demonstrated for ClaI (not shown).

Use of 'Pseudo-sites': The logical extension of the above seems to be that termini of amplicons can be readily adapted to the staggered ends of any given linearized plasmid vector. The minimal match may thereby be restricted just to the overhanging bases with no need to consider adjacent nucleotides (usually an essential part of a restriction site), and hence provide broader versatility.

Faithful enzyme-independent cloning. The ultimate goal of an enzyme-free cloning protocol, which would be suitable for any fragment ends of interest, is easily realized with 'Restriction-PCR'. Most of the critical preconditions for such an application are: (1) A high precision of template copying. The faithful, error-free reconstitution of nucleic acid stretches when combining the single stranded termini. (2) The example depicted in the scheme of Figure 3 impressively demonstrates this fidelity. Two gel-purified plasmid fragments were used as PCR-substrates, which contain in an overlapping fashion two halves of the plasmid pGFPuv, split in a critical region of the AmpR gene (Fig. 3a). The larger fragment 'template A/E', extends from an Asp700 site at pos. 1720 to EcoRI (1008),

and, overlapping, a smaller 'template X/P', from XhoI (710) to PvuI (1956). As each part contained essential pieces of the plasmid's resistance gene, this strategy eliminated any background attributable to contamination with DNA from a viable plasmid had it served as amplification template (Fig. 3a). The non-replicative PCR template fragments 'A/E' and 'X/P' were then individually used for paired "Restriction-PCR" reactions as depicted in Figure 3b. The necessary primers were designed in a way that they would produce an arbitrarily chosen, but matching fifteen-base overhang at the junctions ('jnct.a' and 'jnct.b'), present at either 5' terminus of the final double-stranded fragments after denaturation and re-annealing. Although these synthetic single-stranded overhangs did not resemble any specific restriction site, they should (through their perfect complementarity) allow the precise reconstitution of a complete and functional GFP-expression plasmid. The GFP-expression would indicate frame integrity at the reconstituting DNA-termini. Based on Tm-calculations for the formula [2(A+T)°+4(G+C)°] 15mers, with at least 30% G/C content, will have melting points higher than 37°C. As bacterial incubations following the transformation are done at 37°G, we chose the fifteen-mer overhang as a 'suitable average length' for the single stranded sequence. (For a pure A/T 15mer the Tm is 30°C, for an A/T 19mer: 38°C; a pure G/C 10mer overhang is calculated to melt at 40°C.) The two double-stranded fragment pairs were generated by extension of the oligo pairs no. (oligo 5+oligo 8) plus (oligo 6+oligo 7) on template 'A/E' (Fig. 3b, top part). Stoichiometric mixing, denaturation and re-annealing of the products generated a "half reaction" of 2.3 kbp in size. This final cloning-intermediate possessed a 5'-protruding termini between base pairs 1889-1874 and 821-836, respectively (as drawn in Fig. 3b). In analogy, the 1.1 kbp "half reaction" was generated with (oligo 1+oligo 4) plus (oligo 2+oligo 3) on template 'X/P' with corresponding staggered termini at the identical positions as the fragment above (Fig 3b). For plasmid reconstitution, both size classes were combined at a 1 : 1 ratio and after 15 minutes at room temperature an aliquot was used straight for the bacterial transformation without any ligation step involved. Separate transformation of each "half reaction" served as a negative control in the experiment. Based on the following estimation, relative 'cloning efficiencies' should be a minor concern since one ng of a plasmid of 5 kbp length represents more than 10^6 molecules. The mixing protocol, as described above, will most likely produce several million copies of DNA for the transformation. Therefore, we rather suggest that the quality of competent bacteria will typically have the greatest impact on the cloning success. Moreover, and unlike the T-vector technology, "Restriction-PCR" generates with high precision defined and unique termini for oriented (forced) ligation protocols. Due to this major technical difference, a direct comparison of relative cloning efficiencies with other methodologies was not undertaken. Whereas no bacteria grew on the control plates, the UV-analysis of the main dish revealed 95 percent fluorescent colonies (Figure 3c, where arrows indicate the few non-fluorescent negatives 'neg.'). Acquired mutations in the gene are a likely cause for this rare absence of fluorescence (not determined) as restriction analyses were inconspicuous.

Discussion

The technique of 'Restriction-PCR makes its applicability obvious, as any restriction-site-like DNA-termini can be readily prepared and utilized in whatever cloning applications are required. Ligation-free protocols are of particular value, where e.g. genes of extended length require stepwise cloning in several segments, as they will most likely lack convenient restriction sites. Since numerous molecular applications depend strictly on the sequence authenticity during the DNA amplification, particularly where the function of the DNA to be studied is not well characterized, the use of proofreading polymerases for the essential amplifying PCR is superior to a T-overhang approach. Common approaches that are used to study the need for, or function of certain DNA motifs, use short, internal deletions or insertions at very precise locations of a DNA sequence. Thereby, position and relative distance of putative elements can be preserved in their context. The enzyme-independent "Restriction-PCR" protocol will offer both speed and precision for such a task. One minor disadvantage of the presented technique it is that it requires twice the number of oligonucleotide primers for the cloning procedure. Nevertheless, the gained value outweighs by far the extra effort as synthetic oligonuleotides are now commercially available at very reasonable prices.

Besides a large variety of direct applications, it is conceivable that even the exotic ability to quantitatively generate single stranded mutations, deletions, etc. directly in a double stranded plasmid DNA might have utility.

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