Cloning of PCR Products

Following PCR, it is often necessary to clone the amplified fragment of DNA into a plasmid. The value of cloning an amplicon into a vector depends on the amplicon's future use. Cloning is generally indicated when it constitutes the end-point of a number of complex enzymatic steps (such as those performed in one-sided PCR) or of labor-intensive protocols (such as differential display). Cloning an amplicon avoids having to repeat the reaction every time the product of an amplification is needed. This is particularly important when the template is of limited availability or when the PCR product is difficult to obtain, for example, because of its length. Cloning a PCR product into a vector is also convenient when the amplified DNA fragment will be used as a probe or as a positive control in future PCR procedures. In certain instances, a PCR product may be cloned into a vector suitable for in vivo expression studies. Although expression-PCR (see Section 5) bypasses the need to clone an amplicon for further expression, the desired protein can only be produced by in vitro transcription-translation, not in vivo. A number of strategies are available for cloning PCR products and are described in "Cloning and Analysis of PCR-generated DNA Fragments" and "Strategies for Cloning PCR Products."

A particular cloning strategy should be chosen before the actual PCR is performed, because in a number of approaches, specific sequences need to be added to the 5′ ends of the primers. These added nucleotide sequences are compatible with a particular vector after enzymatic treatment of various types (i.e., T4 DNA polymerase, uracil DNA glycosylase, and restriction enzyme digestion). The cloning strategy will also determine the choice of thermostable DNA polymer-

ase. Two types of thermostable DNA polymerases are available: polymerases with 3′→ 5′ exonuclease (proofreading) activity, and polymerases without this activity. The activities of the thermostable DNA polymerases are shown in Table 1 of the introduction to Section 1. Only enzymes without proofreading activity will add an extra adenosine residue at the 3′ end of a PCR product and therefore should be chosen when cloning the amplicon into vectors that contain overhangs of thymidine residues.

Some cloning strategies provide a means of directionally placing the amplicon within a vector, whereas others are random with respect to amplicon orientation. Directional cloning of a PCR product is beneficial in applications where in vivo and in vitro expression are required.

An important consideration applicable to all cloning methods is the need to optimize the amplification protocol so that the accumulation of spurious DNAs is avoided. These extraneous DNA fragments may compete with the desired PCR product in the ligation reaction, resulting in a reduced number of the desired clones containing the correct insert. To be successful, it may be necessary to add an extra purification step to eliminate these DNA fragments prior to initiating the ligation step.

Strategies for Cloning PCR Products

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INTRODUCTION

Generating PCR products is often only the first step in a series of experiments such as producing specific cDNA clones or making mutations in a particular regulatory sequence or in an open reading frame. Whether the starting material is RNA or DNA, it is important to have an efficient method for cloning PCR products that will facilitate subsequent studies. A variety of methods have been developed to clone the DNA products of PCR amplification (Costa and Weiner 1994a). The method selected to clone PCR fragments is determined by several factors. This might depend on whether the sequence of the PCR product is known, what the unique restriction sites in the vector are, and what will be done with the PCR product; for example, sequencing, mutagenesis, or expression in bacterial or eukaryotic cells.

For some applications, PCR products can be used directly without subcloning. These include sequencing, in vitro transcription, and some mutagenesis studies. For most applications, however, it is essential to subclone a PCR product before further manipulation or analysis. This chapter summarizes several systems available for the preparation and subcloning of PCR products.

After generating a PCR product, it is important to run a small amount of the sample on an agarose gel to verify the size of the product and to ensure that contamination does not exist. For most PCR procedures, it is possible to analyze 5–10% of the product by electrophoresis through agarose and to visualize the DNA by ethidium bromide staining. Depending on the homogeneity of the PCR product, it may be possible to continue directly with cloning.

Several subcloning protocols require that the PCR mix be purified on a column to remove excess primers and nucleotides. This can also be accomplished by doing a selective ammonium acetate precipitation

of the sample. If further enzymatic manipulation is required before cloning, it is recommended that the sample be extracted once or twice with an equal volume of phenol:chloroform/isoamyl alcohol and once or twice with chloroform. After extraction, the sample should be ethanol-precipitated either with sodium acetate (pH 5.5) or with ammonium acetate. Crowe et al. (1991) also describe an increase in cloning efficiency of PCR products if the completed PCR product is first treated with proteinase K and then extracted and precipitated.

If the PCR products are heterogeneous when examined by gel electrophoresis, it is important to gel-purify the desired fragment or fragments before cloning. This can be accomplished by using low-melting-temperature agarose, by electroelution, or by dissolution of the gel and purification of the DNA on glass beads (GENECLEAN Kit, BIO-101 or Elu Quik Kit, Schleicher and Schuell).

Once the sample has been analyzed and, if necessary, purified, the DNA can be subcloned into a bacterial plasmid and used to transform competent bacteria. This chapter describes five methodologies for the cloning of PCR products, several of which are facilitated by the availability of commercially prepared cloning vectors. These methods are restriction endonuclease site incorporation (Kaufman and Evans 1990; Scharf 1990; Lorens 1991), uracil DNA glycosylase (UDG) cloning (Duncan 1981; Friedberg et al. 1981; Nisson et al. 1991; Buchman 1992, 1993), ligation-independent cloning (LIC-PCR) (Aslanidis and de Jong 1990; Shuldiner et al. 1990; Haun et al. 1992), T/A cloning (Clark 1988; Holton and Graham 1991; Marchuk et al. 1991; Mead et al. 1991; Hu 1993), and blunt-end cloning (Liu and Schwartz 1992; Weiner 1993; Costa and Weiner 1994a,b; Costa et al. 1994). With the exception of restriction endonuclease site incorporation, all of these techniques rely on the use of a specific vector in which to ligate the PCR product. The construction and availability of these vectors are described.

RESTRICTION ENDONUCLEASE SITE INCORPORATION

One of the primary methods for cloning a PCR product is to digest the product with restriction endonucleases whose recognition sequences are present in the amplified DNA (Scharf 1990). Cloning of the digested product is straightforward and no special reagents or vectors are required. For some applications, where the sequence of the PCR product is known, it may be possible to utilize restriction endonuclease sites within the DNA to clone the PCR product. However, it is also possible to incorporate restriction endonuclease sites into the product by designing primers that contain specific or unique restriction sites at their 5′ termini. The resulting PCR product will be flanked by the same restriction endonuclease site at both termini or by different sites at either end. Digestion of the DNA with the appropriate enzyme or enzymes will yield an insert with compatible

ends to the desired vector. The vector and insert DNAs are then ligated and transformed into competent bacteria.

Several factors must be considered in designing PCR primers that contain restriction endonuclease sites. First, if the sequence of the PCR product is not known, the product DNA may contain one of the sites utilized in the primers. This would lead to internal cleavage of the product, giving rise to deleted clones. Several restriction endonucleases are now available which have 8-bp recognition sequences (*Asc* I, *Not* I, *Pac* I, *Pme* I, *Sfi* I, *SgrA* I, *Srf* I, *Sse8387* I, and *Swa* I). It is suggested that these enzyme sites be used for cloning when the sequence of the template RNA or DNA is not known. The frequency with which these enzymes cleave DNA is significantly lower than those with recognition sequences of six or less base pairs.

A second consideration when using restriction endonuclease site incorporation as a method for cloning PCR products is the efficiency with which the desired enzyme will cleave the DNA product, if the enzyme recognition site is near the terminus of the DNA. The table on page 542 shows the efficiency of enzyme digestion for various restriction endonucleases when the substrate DNA is a short region. As shown, enzymes vary considerably in their ability to cleave small fragments of DNA. To increase the efficiency of restriction endonuclease digestion of PCR products, it is important to incorporate several extra nucleotides at the 5′ end of the primer. This table can serve as a guide for the number of nucleotides required in addition to any particular restriction endonuclease recognition sequence to ensure efficient digestion. Kaufman and Evans (1990) have also described the efficiency of restriction endonuclease cleavage of restriction sites in the polylinker of the pBluescript II (Stratagene) cloning vector.

Lorens (1991) has described a technique that circumvents the difficulties associated with inefficient cleavage due to a restriction endonuclease site too close to the end of the DNA product. Briefly, the procedure utilizes Klenow, T4 polynucleotide kinase, and T4 DNA ligase. By kinasing the PCR product, filling in any overhangs, and ligating, a concatamer of PCR products is formed. This multimer can then be efficiently cleaved by the desired restriction endonuclease. The linear product is purified and ligated into a vector with compatible ends.

A third consideration when using this technique for cloning PCR products is that each restriction endonuclease has a specific requirement for salt. It is therefore necessary to purify the PCR product on a column, or by extracting and precipitating the DNA before digesting. This ensures that the buffer from the PCR does not interfere with restriction endonuclease digestion. This also prevents the polymerase present in the PCR from filling in the overhangs created by restriction endonuclease digestion. It is important to gel-purify, or to re-extract

the sample after digestion to remove the restriction endonuclease. This will increase the efficiency of insert ligation with the vector DNA.

The use of restriction endonuclease site incorporation to clone PCR products requires several additional steps after PCR. Each additional

Cleavage close to the End of DNA Fragments

ENZYME	OLIGO SEQUENCE	CHAIN LENGTH	% CLEAVAGE 2 HR	20 HR
Acc I	GGTCGACC	8	0	0
	CGGTCGACCG	10	0	0
	CCGGTCGACCGG	12	0	0
Afl III	CACATGTG	8	0	0
	CCACATGTGG	10	>90	>90
	CCCACATGTGGG	12	>90	>90
Asc I	GGCGCGCC	8	>90	>90
	AGGCGCGCCT	10	>90	>90
	TTGGCGCGCCAA	12	>90	>90
Ava I	CCCCGGGG	8	50	>90
	CCCCCGGGGG	10	>90	>90
	TCCCCCGGGGGA	12	>90	>90
BamH I	CGGATCCG	8	10	25
	CGGGATCCCG	10	>90	>90
	CGCGGATCCGCG	12	>90	>90
Bgl II	CAGATCTG	8	0	0
	GAAGATCTTC	10	75	>90
	GGAAGATCTTCC	12	25	>90
BssH II	GGCGCGCC	8	0	0
	AGGCGCGCCT	10	0	0
	TTGGCGCGCCAA	12	50	>90
BstE II	GGGT(A/T)ACCC	9	0	10
BstX I	AACTGCAGAACCAATGCATTGG	22	0	0
	AAAACTGCAGCCAATGCATTGGAA	24	25	50
	CTGCAGAACCAATGCATTGGATGCAT	27	25	>90
Cla I	CATCGATG	8	0	0
	GATCGATC	8	0	0
	CCATCGATGG	10	>90	>90
	CCATCGATGGG	12	50	50
EcoR I	GGAATTCC	8	>90	>90
	CGGAATTCCG	10	>90	>90
	CCGGAATTCCGG	12	>90	>90
Hae III	GGGGCCCC	8	>90	>90
	AGCGGCCGCT	10	>90	>90
	TTGCGGCCGCAA	12	>90	>90
Hind III	CAAGCTTG	8	0	0
	CCAAGCTTGG	10	0	0
	CCCAAGCTTGGG	12	10	75
Kpn I	GGGTACCC	8	0	0
	GGGGTACCCC	10	>90	>90
	CGGGGTACCCCG	12	>90	>90
Mlu I	GACGCGTC	8	0	0
	CGACGCGTCG	10	25	50
Nco I	CCCATGGG	8	0	0
	CATGCCATGGCATG	14	50	75
Nde I	CCATATGG	8	0	0
	CCCATATGGG	10	0	0
	CGCCATATGGCG	12	0	0
	GGGTTTCATATGAAACCC	18	0	0
	GGAATTCCATATGGAATTCC	20	75	>90
	GGGAATTCCATATGGAATTCCC	22	75	>90
Nhe I	GGCTAGCC	8	0	0
	CGGCTAGCCG	10	10	25
	CTAGCTAGCTAG	12	10	50

ENZYME	OLIGO SEQUENCE	CHAIN LENGTH	% CLEAVAGE 2 HR	20 HR
Not I	TTGCGGCCGCAA	12	0	0
	ATTTGCGGCCGCTTTA	16	10	10
	AAATATGCGGCCGCTATAAA	20	10	10
	ATAAGAATGCGGCCGCTAAACTAT	24	25	90
	AAGGAAAAAAGCGGCCGCAAAAGGAAAA	28	25	>90
Nsi I	TGCATGCATGCA	12	10	>90
	CCAATGCATTGGTTCTGCAGTT	22	>90	>90
Pac I	TTAATTAA	8	0	0
	GTTAATTAAC	10	0	25
	CCTTAATTAAGG	12	0	>90
Pme I	GTTTAAAC	8	0	0
	GGTTTAAACC	10	0	25
	GGGTTTAAACCC	12	0	50
	AGCTTTGTTTAAACGGCGCCGG	24	75	>90
Pst I	GCTGCAGC	8	0	0
	TGCACTGCAGTGCA	14	10	10
	AACTGCAGAACCAATGCATTGG	22	>90	>90
	AAAACTGCAGCCAATGCATTGGAA	24	>90	>90
	CTGCAGAACCAATGCATTGGATGCAT	26	0	0
Pvu I	CCGATCGG	8	0	0
	ATCGATCGAT	10	10	25
	TCGCGATCGCGA	12	0	10
Sac I	CGAGCTCG	8	10	10
Sac II	GCCGCGGC	8	0	0
	TCCCCGCGGGGA	12	50	>90
Sal I	GTCGACGTCAAAAGGCCATAGCGGCCGC	28	0	0
	GCGTCGACGTCTTGGCCATAGCGGCCGCGG	30	10	50
	ACGCGTCGACGTCGGCCATAGCGGCCGCGGAA	32	10	75
Sca I	GAGTACTC	8	10	25
	AAAAGTACTTTT	12	75	75
Sma I	CCCGGG	6	0	10
	CCCCGGGG	8	0	10
	CCCCCGGGGG	10	10	50
	TCCCCCGGGGGA	12	>90	>90
Spe I	GACTAGTC	8	10	>90
	GGACTAGTCC	10	10	>90
	CGGACTAGTCCG	12	0	50
	CTAGACTAGTCTAG	14	0	50
Sph I	GGCATGCC	8	0	0
	CATGCATGCATG	12	0	25
	ACATGCATGCATGT	14	10	50
Stu I	AAGGCCTT	8	>90	>90
	GAAGGCCTTC	10	>90	>90
	AAAAGGCCTTTT	12	>90	>90
Xba I	CTCTAGAG	8	0	0
	GCTCTAGAGC	10	>90	>90
	TGCTCTAGAGCA	12	75	>90
	CTAGTCTAGACTAG	14	75	>90
Xho I	CCTCGAGG	8	0	0
	CCCTCGAGGG	10	10	25
	CCGCTCGAGCGG	12	10	75
Xma I	CCCCGGGG	8	0	0
	CCCCCGGGGG	10	25	75
	CCCCCCGGGGGG	12	50	>90
	TCCCCCCGGGGGGA	14	>90	>90

To test the ability of a restriction endonuclease to cleave a site that lies within a few bases of the end of a DNA fragment, a series of short, double-stranded oligonucleotides that contain the restriction endonuclease recognition site were digested. The table above illustrates the varying requirements restriction endonucleases have for the number of bases flanking their recognition sequences. Enzyme recognition sites appear in bold print. (Reprinted, with permission, from the 1993/94 New England Biolabs Catalog.)

step takes time and can lead to a loss in DNA recovery, potentially reducing cloning efficiency. Several of the cloning methods available for inserting PCR products into plasmids are optimized for speed and efficiency. They require as little manipulation as possible after the completion of the PCR. These methods, ligation-independent cloning, blunt-end cloning, and T/A cloning, do not require extraction and precipitation of the PCR products or lengthy enzyme digestion of the vector or the insert.

CLONING WITHOUT LIGATION

Several methods are available for cloning PCR products without having to perform a ligation reaction. These methods increase the efficiency of insertion of the amplified DNA into the vector and do not require a lengthy ligation reaction. The PCR products to be cloned contain long single-stranded tails, usually ≥12 bases, that are annealed to a linearized vector that contains complementary single-stranded tails. Because of the length of the corresponding complementary overhangs, no ligase reaction is required. After annealing, the vector and insert are transformed directly into competent bacteria. It is possible to generate either symmetrical or asymmetrical overhangs for either bidirectional or unidirectional cloning, respectively, depending on the intended use of the PCR product. Several methods are available for generating single-stranded tails that are long enough to form a stable interaction between the vector and the insert such that ligation is not required for transformation. These are uracil DNA glycosylase (UDG) cloning (Duncan 1981; Friedberg et al. 1981; Nisson et al. 1991; Buchman et al. 1992, 1993), ligation-independent cloning (Aslanidis and de Jong 1990; Haun et al. 1992), and PCR-induced subcloning (Shuldiner et al. 1990).

UDG Cloning

UDG cloning takes advantage of the biological properties of UDG, an enzyme involved in the TTP biosynthetic pathway that cleaves the *N*-glycosylic bond between the deoxyribose moiety and uracil. This cleavage yields abasic dU residues, which lead to a disruption of DNA base pairing (Duncan 1981; Friedberg et al. 1981). To utilize this enzyme for the cloning of PCR products, primers must be designed that contain dUMP in the 5′-terminal nucleotides. These nucleotides correspond to the complementary overhang in the vector sequences (Nisson et al. 1991). UDG cleaves the incorporated UMP residues in the final PCR products and generates a single-stranded 3′ overhang. It is important to generate a 3′ tail that contains a sufficient number of nucleotides so that efficient and stable annealing occurs between the vector and PCR insert DNAs. It is also important to design primers such that a dUMP is inserted at the junction between the sequences

complementary to the vector and the PCR template sequences, as well as to incorporate several dUMP residues into the 5′-terminal nucleotides of the oligonucleotide primer so that after cleavage with UDG, the other base-paired oligonucleotides lose contact with the opposite strand, thereby generating a single-stranded overhang.

To generate unique vector- and insert-specific overhangs, it is necessary to design primers for the cloning vector that are complementary to the overhangs generated for the insert fragment. These primers are then used to PCR-amplify and generate a linearized vector with compatible overhangs. In this method, both the vector and the insert are treated with UDG, annealed, and transformed into bacteria (Nisson et al. 1991). The advantage of synthesizing unique vector- and insert-specific overhangs is the ability to customize the nucleotides in the primers to contain sequences specific to a template DNA, or the incorporation of specific restriction sites that allow further manipulation after the PCR product is cloned. The disadvantages of generating both vector- and insert-specific primers are the requirement for two additional oligonucleotide primers for the vector, and the fact that mistakes in the PCR amplification of the vector may lead to alterations of important vector sequences, such as the *lacZ* gene, the bacterial antibiotic resistance gene, the origin of replication, or other regulatory sequences in the vector, such as eukaryotic promoters. These mutations can lead to a decrease in the cloning efficiency and cause problems with subsequent uses of the plasmid.

Several vectors are commercially available (Life Technologies) which facilitate the cloning of products that have dUMP incorporated into the 5′-terminal nucleotides on both strands. The available vectors are CLONEAMP pAMP 1, CLONEAMP pAMP 10, CLONEAMP pAMP 18, and CLONEAMP pAMP 19 (Fig. 1A). These vectors are provided as linear DNA molecules with defined 3′ overhangs, and to use these vectors, all oligonucleotide primers must have the appropriate complementary nucleotides at the 5′ end (Fig. 1B). As described above, after PCR, the products are treated with UDG in the presence of vector, annealed for 30 minutes at 37°C, and then transformed into competent bacteria (Fig. 1C).

Figure 1 shows the important features for each of these vectors, sequences at the polylinker cloning site, and a schematic diagram of the UDG cloning protocol. CLONEAMP pAMP 1 has asymmetrical 12-base overhangs, 5′-CTACTACTACTA-3′ and 5′-CATCATCATCAT-3′, which are nested within a polylinker region flanked by either the SP6 or T7 phage promoters. CLONEAMP pAMP 10 is identical to CLONEAMP pAMP 1 except that the cloning region comprises two symmetrical 12-nucleotide overhangs, 5′-CTACTACTACTA-3′. Inserts anneal in both orientations in the CLONEAMP pAMP 10 vector. CLONEAMP pAMP 18 and CLONEAMP pAMP 19 are identical to

pUC 18 and pUC 19, respectively, except for the addition of several new restriction endonuclease sites within the polylinker region that make up the single-stranded cloning region (Fig. 1A). These vectors are provided as linear DNA with 12-base overhangs generated within the polylinker region. Figure 1B shows the 5′-terminal primer sequences needed for each of the vectors.

Ligation-Independent Cloning

The second system for cloning that does not require ligation is based on generating PCR products that have one of the dNTPs missing in the 5′-terminal nucleotides (Aslanidis and de Jong 1990; Haun et al. 1992). This is accomplished by designing primers that have only three of the nucleotides present in the first 12 or more bases, depending on the desired length of the overhang. After generating a PCR product, the sample is treated with T4 DNA polymerase in the presence of the dNTP, which is not present in the 5′-terminal sequences. T4 DNA polymerase has exonuclease activity as well as polymerase activity. In the absence of the necessary dNTPs, the exonuclease activity removes nucleotides in a 5′→ 3′ direction from the ends of the PCR product until the enzyme reaches the first nucleotide in the amplified DNA that corresponds to the dNTP present in the reaction mix. The presence of this dNTP inhibits further exonuclease activity, and the single-stranded 3′ overhang is stable. The enzymatic activity is killed by heat-inactivation and the insert is annealed to a linearized vector treated in a similar way and transformed into competent bacteria.

A commercially available vector, pDIRECT (CLONTECH), has been modified for the ligation-independent cloning (LIC) of products that have been treated with T4 DNA polymerase. Figure 2 shows a schematic map of the vector, its polylinker, and the single-stranded region for annealing with insert DNA (Fig. 2A), as well as a diagram of the LIC protocol (Fig. 2B). The single-stranded ends of the DNA are asymmetrical, 5′-GGGCCGAACCAG-3′ and 5′-GGGCGAGCGAG-3′, and therefore the insert is cloned unidirectionally. This vector has the T3 and T7 phage promoters flanking the cloning region to allow direct sequencing of the insert or in vitro transcription of the insert from either strand.

When using this vector, it is essential to design PCR primers that contain the specific nucleotide sequence that corresponds to the complementary sequences of the pDIRECT vector. In this vector, there are no T residues in the single-stranded region; therefore, dTTP must be included in the T4 DNA polymerase reaction to block exonuclease activity at the first T in the vector or in the insert. This vector is supplied as a linear DNA with the appropriate buffers and T4 DNA polymerase. As described above, after treatment of the PCR products with T4 DNA

polymerase, the vector and insert are annealed and then transformed into competent bacteria. The cloning site is within the *lacZ* gene, and a color selection can be used to identify clones that are positive for the insert DNA.

PCR-induced Subcloning

The third ligation-independent method for cloning PCR products is described by Shuldiner et al. (1990). This strategy is called PCR-induced (ligase-free) subcloning and utilizes primers that have 24 additional nucleotides at the 5′ end that correspond to the 3′ ends of the linearized vector. After initial amplification, the PCR product is divided into two tubes and mixed with linearized vector. A second set of PCR amplifications is performed using the 24 vector-specific bases in the original PCR product as the primer at one end and a second set of primers, one in each reaction, that correspond to the complementary strands at the other termini of the vector. The second PCR results in the generation of two full-length vector- and insert-containing clones, one with the insert upstream of the vector and one with the insert downstream from the vector. The two samples are mixed, chemically denatured, and then reannealed. The annealed product contains a long region of overlap and can therefore be transformed directly into competent bacteria. The advantages of this sys-

Figure 1 CLONEAMP pAMP vectors and the UDG cloning strategy. (*A*) Schematic maps of pAMP vectors. pAMP 1 and pAMP 10 are identical vectors except for the sequence of the single-stranded region (shown here, and described in the text) required for annealing with insert DNA. pAMP 1 is for unidirectional cloning of insert DNA and pAMP 10 is for bidirectional cloning. pAMP 1 and pAMP 10 contain a bacterial origin of replication, the ampicillin resistance gene for the selection of transformed bacteria, and the f1 intergenic region from M13 phage for the production of single-stranded DNAs. The polylinker is within the *lacZ* gene, permitting color selection of positive colonies. Vectors pAMP 18 and pAMP 19 are modified pUC18 and pUC19 vectors. They have a single-stranded region added in the polylinker of the original vectors for annealing to complementary PCR product inserts. The single-stranded cloning region contains the restriction endonuclease sites *NcoI* and *BglII* on one strand, and *MluI* and *SpeI* on the other strand. The two plasmids are identical except for the orientation of the polylinker region (shown here), which is inverted. The single-stranded tails are the same for both clones and can be used for directional cloning of an insert. The vectors contain a bacterial origin of replication and the ampicillin resistance gene for the selection of transformed bacteria. The polylinker, within the *lacZ* gene, permits color selection of positive colonies. (*B*) dUMP PCR primer tails for pAMP 1, pAMP 10, pAMP 18, and pAMP 19. The sequences shown for each clone are required at the 5′ end of oligonucleotide primers to be used for PCR amplification. (*C*) pAMP cloning protocol. PCR primers with pAMP-specific sequences at the 5′ end are used to amplify DNA. PCR products are treated with UDG in the presence of a compatible pAMP vector (supplied as a linear molecule). The vector and the insert are then annealed, and the recombinant plasmid is transformed into competent bacteria. Primer-specific sequences for pAMP 1 are shown in this diagram. The protocol is identical for all of the pAMP clones. (Adapted, with permission, from Life Technologies Inc.)

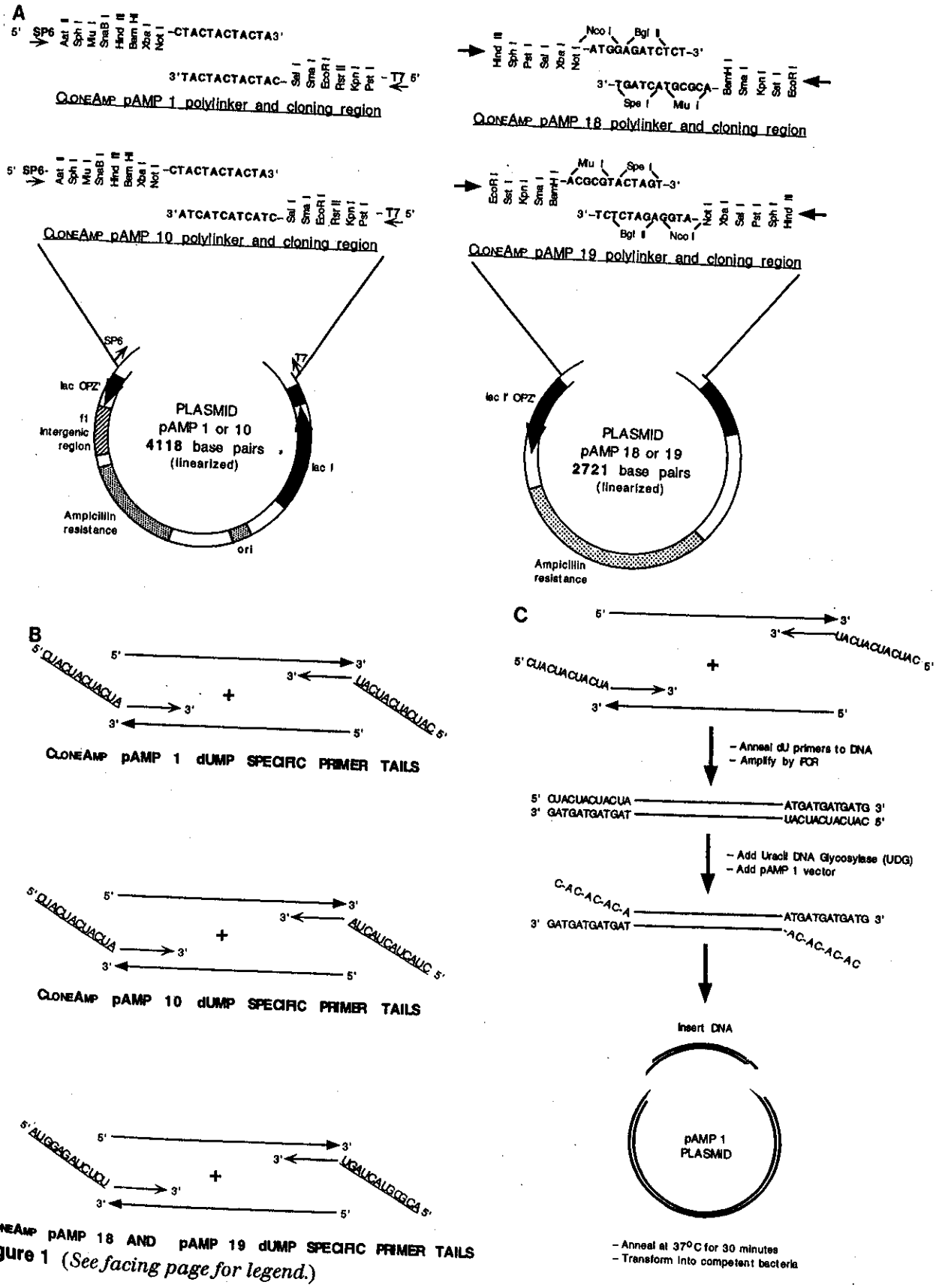

Figure 1 *(See facing page for legend.)*

tem are that any vector can be used and the cloning is directional. However, although this procedure does not require any enzymatic alteration of the PCR products, it does require the production of very long oligonucleotide primers for the first PCR amplification and an additional set of primers for the secondary PCR. Also, because the vector is being reamplified, the potential problems associated with misincorporation, as described above, apply.

T/A CLONING

One method for the direct cloning of PCR products utilizes linearized vectors that contain a single 3′ thymidine (T) overhang and inserts

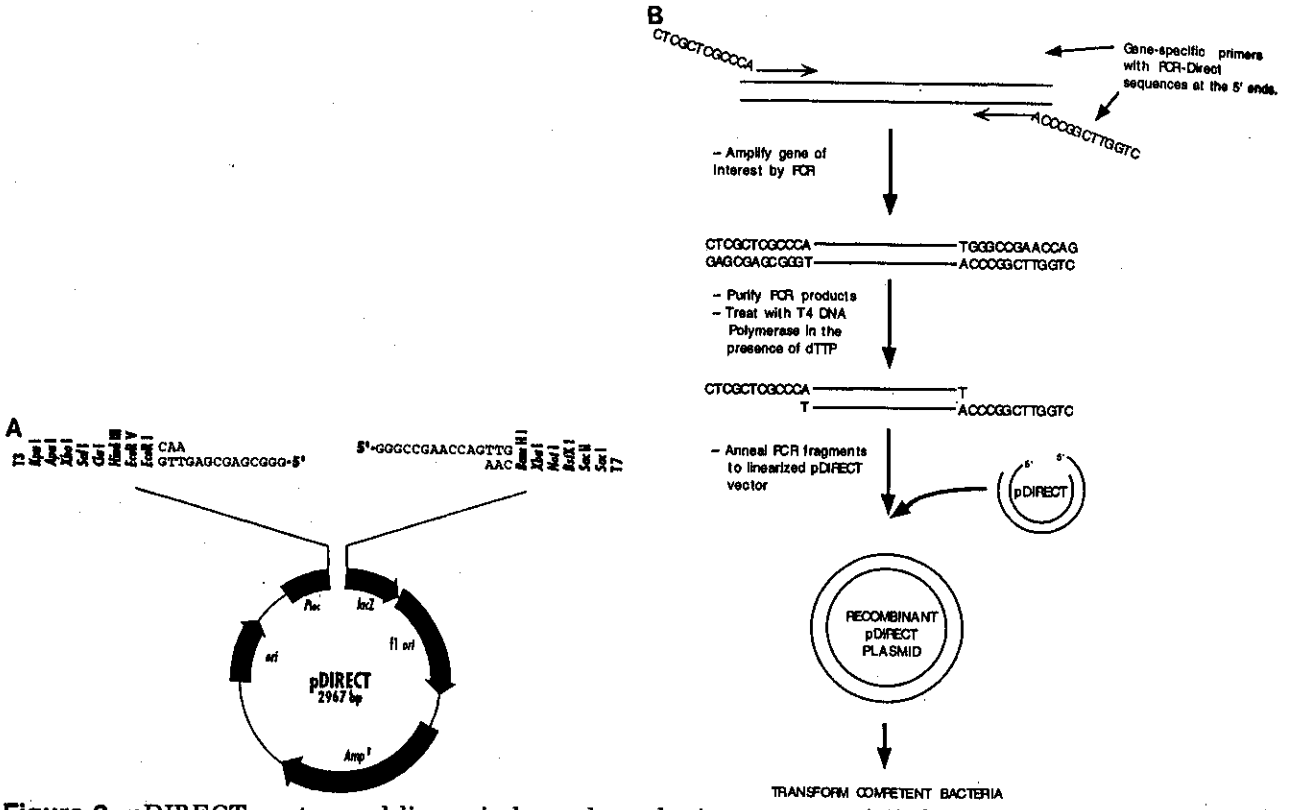

Figure 2 pDIRECT vector and ligase-independent cloning strategy. (*A*) Schematic map of pDIRECT. The linearized pDIRECT vector contains noncomplementary 5′ single-stranded ends for cloning PCR products using the PCR-Direct system. In addition, pDIRECT contains the f1 origin of replication for the synthesis of single-stranded DNA upon coinfection with M13 helper phage. The cloning site and the multiple restriction enzyme sites flanking it are located in the *lacZ* gene, allowing blue/white color screening for recombinant plasmids. T3 and T7 promoters flank the cloning site in opposite orientations. (*B*) Schematic diagram of pDIRECT cloning procedure. PCR primers with PCR-Direct sequences at the 5′ end are used to amplify the desired DNA. The PCR product is purified and then treated with T4 DNA polymerase in the presence of dTTP. The PCR insert fragment is annealed with the pDIRECT vector, which is supplied as a linear DNA molecule, and the recombinant plasmid is used to transform competent bacteria. (Adapted, with permission, from CLONTECH Laboratories, Inc. [PCR Research Tools Catalog].)

with a single 3′ adenosine (A) overhang. This system is called T/A cloning and takes advantage of the extendase activity that several of the DNA polymerases have (Clark 1988; Hu 1993). Extendase activity is defined as the non-template-dependent addition of a single nucleotide at the 3′ end of an extended PCR product. For *Thermus aquaticus*, *Thermus flavus*, and *Thermococcus litoralis*, this nucleotide is generally an A residue; however, the added nucleotide differs according to the terminal nucleotide in the template-dependent product (Hu 1993).

Several techniques are available for generating linear vectors that have a single 3′ T overhang so that the PCR products containing a 3′ A residue can be directly ligated into the vectors. One method uses vectors that have been linearized with enzymes that leave a single T nucleotide overhang (Mead et al. 1991). Three such restriction endonucleases are available: *Mbo*II, *Xcm*I, and *Hph*I. *Hph*I and *Mbo*II each have a 5-bp recognition sequence and a cleavage site 8 nucleotides downstream that generates a single 3′ nucleotide overhang. *Xcm*I has a 15-bp recognition sequence that is cleaved internally to generate a single-base overhang. It is possible to design each of these restriction sites so that the single-base overhang is a T residue. It is essential to insert two inverted copies of the restriction site in the vector, such that a T residue is present on both strands after digestion.

Two additional methods are available for generating vectors with a single 3′ T residue tail. These rely on the addition of a single T residue to a vector that has been linearized by a restriction enzyme that generates a blunt end (Holton and Graham 1991; Marchuk et al. 1991). The first method adds a dideoxy-thymidine triphosphate (ddTTP) using terminal transferase. Because a dideoxy-nucleotide is used, only a single T residue is added to the vector template (Holton and Graham 1991). The presence of a ddTMP nucleotide at the 3′ ends of the vector does not inhibit ligation of the vector with the PCR product. The second method takes advantage of the extendase activity described for *Taq* DNA polymerase (Marchuk et al. 1991). *Taq* DNA polymerase preferentially adds an A residue to the 3′ end of DNA templates; however, the polymerase does add other nucleotides at a lower efficiency. To add a single 3′ T residue, the blunt-end vector is incubated with *Taq* polymerase in the presence of high levels of dTTP. In the absence of any other nucleotide, a single 3′ T residue is added to the vector, generating compatible ends for ligation with PCR products containing an extra 3′ A residue.

Several commercially available vectors (Invitrogen) are designed to facilitate the cloning of PCR products containing an additional 3′ A overhang. The vectors are called T/A cloning vectors (Fig. 3) and are supplied as linear DNAs with a single 3′ T nucleotide overhang on each strand. PCR products can be ligated directly into the vectors

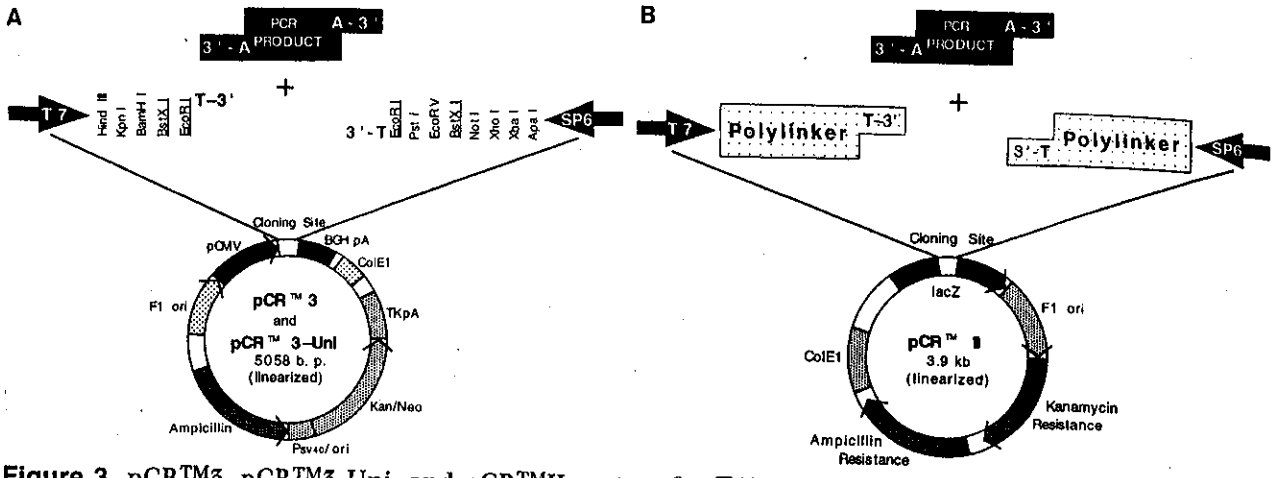

Figure 3 pCR™3, pCR™3-Uni, and pCR™II vectors for T/A cloning. (*A*) Vector map of pCR™3 and pCR™3-Uni. These two vectors are identical, except that pCR™3-Uni has been modified so that the linear vector is hemiphosphorylated. This allows the directional cloning of PCR product DNAs that are also hemiphosphorylated. The polylinker and T/A cloning sites in these vectors are downstream from the immediate-early CMV promoter to allow the direct expression of the DNA insert in eukaryotic cells. Downstream from the cloning site is the bovine growth hormone (BGH) polyadenylation signal. These vectors also contain the neomycin/kanamycin resistance gene, under the control of the SV40 early promoter, for the selection of stable, G418-resistant, eukaryotic cell lines. Other features of these vectors are the bacterial origin of replication, the ampicillin and kanamycin resistance genes for the selection of transformed bacteria, the f1 origin from M13 phage for the production of single-stranded DNA, and the T7 and SP6 phage promoters flanking the cloning site for the in vitro transcription of sense and antisense strands. These vectors are supplied as linear molecules and the PCR product is ligated directly into these vectors without modifications. (*B*) Vector map of pCR™II. Vector pCR™II contains a bacterial origin of replication, the ampicillin and kanamycin resistance genes for the selection of transformed bacteria, the f1 origin from M13 phage for the production of single-stranded DNA, and the T7 and SP6 phage promoters flanking the cloning site for the in vitro transcription of sense and antisense strands. The polylinker region is in the *lacZ* gene, allowing blue/white colony selection to identify positive clones. pCR™II is supplied as a linear molecule, and the PCR product is ligated directly into this vector without modifications. (Adapted, with permission, from Invitrogen Corporation.)

without further enzymatic modification, or if necessary, the insert DNA can be gel-purified before ligation.

Two vectors, pCR™3 and pCR™3-Uni (Fig. 3A), are designed as eukaryotic expression vectors. In addition to the T/A cloning site and polylinker region, these vectors contain the immediate-early cytomegalovirus (CMV) promoter upstream of the cloning site. This promoter directs efficient transcription in most eukaryotic cell lines. Downstream from the cloning site is the bovine growth hormone (BGH) poly(A) signal and the transcription termination signal. This sequence allows the efficient polyadenylation of the expressed open reading frame (ORF) in eukaryotic cells and aids message stability. These two vectors also contain the SV40 promoter/origin upstream of the neomycin/kanamycin resistance gene for the selection of stable

cell lines expressing the inserted ORF. The SV40 origin directs episomal replication in cell lines expressing the SV40 large T antigen.

The vector pCR™3-Uni has been modified so that the 5′ phosphate has been removed from the upstream, or left, arm of the vector. To ensure that the insert is ligated in only one orientation, the upstream or forward primer must be phosphorylated at the 5′ end. Using this primer and an unphosphorylated downstream, or reverse, primer, a hemiphosphorylated PCR product is generated. Because the vector and the insert each have only one strand with a 5′ phosphate, the insert only ligates in one orientation. Although the cloning efficiency is reduced, more than 90% of the plasmids with inserts have the PCR product in the correct orientation.

The second T/A cloning vector, pCR™II (Fig. 3B), has a polylinker surrounding the T/A cloning site that is flanked by the SP6 and T7 phage promoters for in vitro transcription and for sequencing of the inserts. The polylinker region for this vector is in the *lacZ* gene, so that a color selection can be used to identify clones with inserts. The vector contains both the ampicillin and the kanamycin resistance genes for the selection of vector-containing bacteria.

BLUNT-END CLONING

A method for cloning PCR fragments that does not rely on the addition of specific "primer-tails" or the incorporation of restriction endonuclease sites at the 5′ ends of the oligonucleotide primers is the direct ligation of PCR products into vectors linearized with a restriction endonuclease that generates a blunt end. This method is more inefficient than other cloning procedures, because the ligation of blunt ends favors the direct recircularization of the vector; however, there are several methods that increase the efficiency of insert ligation. One is to have a large molar excess of insert in the ligation reaction. A second method to increase the efficiency of blunt-end ligation is to treat the vector with alkaline phosphatase to remove the 5′ phosphates from both ends of the vector. This generates termini that do not serve as substrates for T4 DNA ligase and therefore prevent recircularization of the vector. When ligating a PCR product with a phosphatased vector, it is essential to generate PCR products that are phosphorylated at both 5′ ends. This can be achieved by directly kinasing the amplified DNA or by using primers that have been kinased or have a phosphorylated nucleotide incorporated at the 5′ end.

Another important factor that leads to the inefficient cloning of unmodified PCR products is the incorporation of a single nucleotide (usually an A residue) at the 3′ end of the amplified PCR products. Several of the thermostable DNA polymerases have template-independent extendase activity that incorporates an additional nucleotide at the 3′ end of the DNA product (Clark 1988; Hu 1993). Products that contain an extra nucleotide ligate with a very low ef-

ficiency into blunt-end vectors. Some cloning strategies, described above, take advantage of the extendase activity of DNA polymerases, but for blunt-end cloning, it is important to use a thermostable DNA

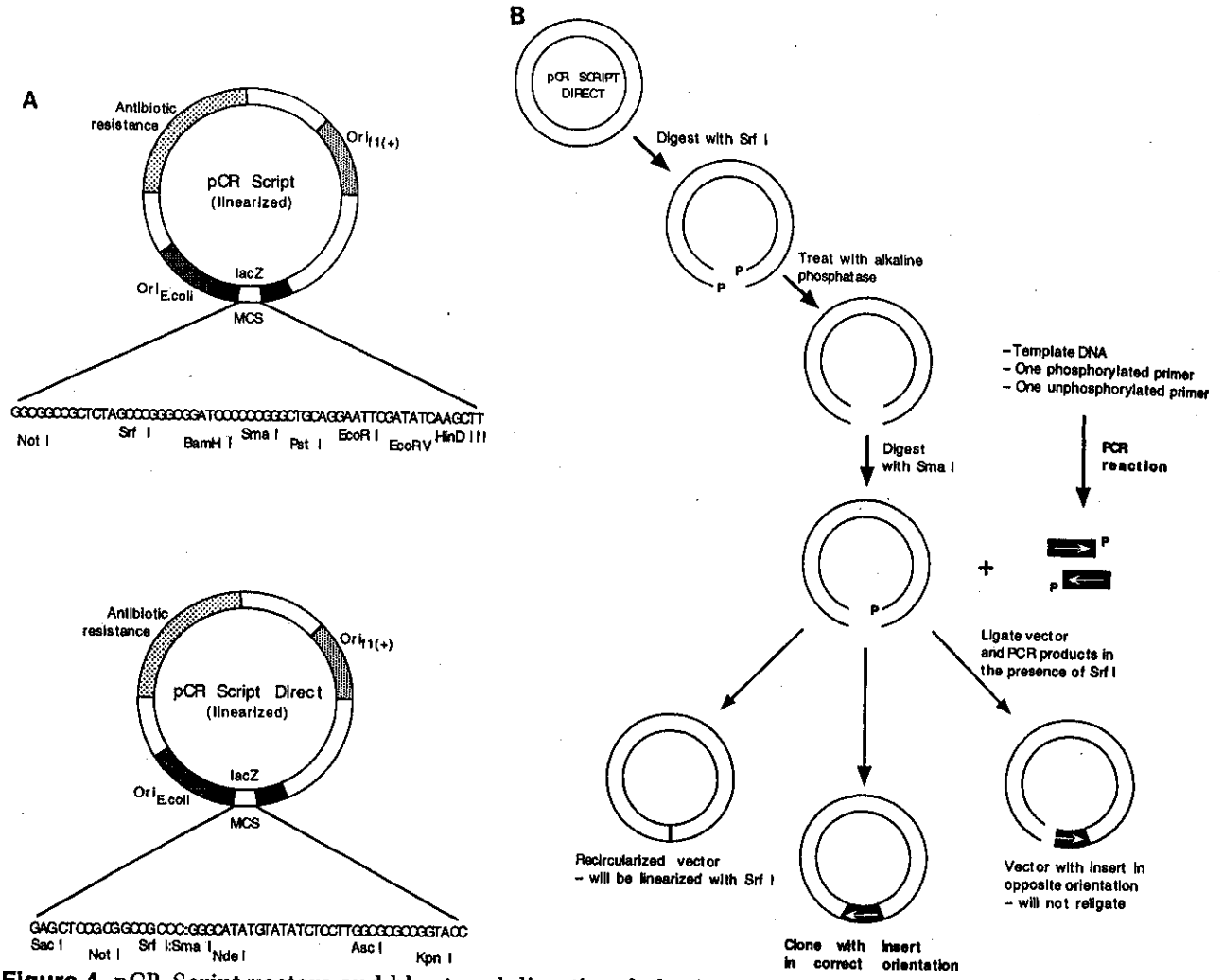

Figure 4 pCR-Script vectors and blunt-end directional cloning strategy. (*A*) Vector maps of pCR-Script and pCR-Script Direct. The pCR-Script vectors contain a bacterial origin of replication, an antibiotic resistance gene for selection of transformed bacteria, and the f1 origin from M13 phage for the synthesis of single-stranded DNA. These vectors differ in the restriction endonuclease sites in the polylinker region. The polylinker region is within the *lacZ* gene, allowing blue/white color selection of positive colonies. pCR-Script Direct has been modified so that the linear DNA is hemiphosphorylated. DNA inserts that are also hemiphosphorylated ligate in one orientation into this vector. (*B*) pCR-Script Direct cloning protocol. This diagram shows the steps involved in the preparation of pCR Script Direct and the ligation with insert DNA. After digestion with restriction endonuclease *Srf* I, the vector is treated with alkaline phosphatase to remove 5′ phosphates from the ends of the DNA. The vector is subsequently digested with restriction endonuclease *Sma*I, generating a blunt-end product that contains a single 5′ phosphate. This vector, supplied as a linear molecule, is ligated directly with a hemiphosphorylated PCR product. The recombinant plasmid is then transformed into competent bacteria. (Adapted, with permission, from Costa and Weiner 1994b [Stratagene Cloning Systems].)

polymerase that has proofreading activity, and therefore reduced extendase activity, or to remove any extra nucleotides from the PCR product. This can be accomplished by incubating the amplified DNA with a DNA polymerase that has exonuclease activity. If the reaction is done in the presence of the four deoxynucleotide triphosphates (dNTPs), only the extra 3′ nucleotide is removed, generating a blunt-end product (Liu and Schwartz 1992). Two DNA polymerases, T4 DNA polymerase and *Pfu* polymerase (from *Pyrococcus furiosus*), are recommended for removing terminal 3′ nucleotides from PCR products (Costa and Weiner 1994b).

Two commercially available vectors, pCR Script and pCR Script Direct (Stratagene), have been optimized for cloning blunt-end PCR products (Costa and Weiner 1994a; Costa et al. 1994b). Inserts are ligated bidirectionally into pCR Script plasmids and unidirectionally into pCR Direct. Figure 4 shows a schematic diagram of the two vectors (Fig. 4A) and a diagram of the protocol for using the pCR Script Direct clone (Fig. 4B). The next chapter in this section ("Cloning and Analysis of PCR-generated DNA Fragments) describes in detail the basis for this blunt-end cloning protocol.

Each of the techniques described here has different advantages and disadvantages. The method chosen for cloning PCR products depends on the type and amount of PCR product being generated, the ultimate use of the PCR product, and the resources available to the researcher. Under normal reaction conditions, a large amount of PCR product is produced and the efficiency of obtaining clones, regardless of the cloning procedure used, is very high. It becomes necessary to optimize the cloning of PCR products when very small amounts of insert DNA are generated. For each of the methodologies described here, it is important to optimize conditions to ensure the generation of clones with the correct insert, which can then be used for further studies.

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