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Mini-Review

Strategies for cloning unknown cellular flanking DNA sequences from foreign integrants

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Abstract. Many virus and transposon DNAs can integrate into the host genome. In this review, techniques, including inverse polymerase chain reaction (IPCR), novel Alu-PCR and vectorette- or splinkerette-PCR are introduced as possible strategies for cloning flanking DNA regions of the integrants. Targeted gene-walking PCR, restriction-site PCR, capture PCR, and panhandle PCR and boomerang DNA amplification are also described. The principles, advantages and limitations of each approach are discussed.

Key words. Boomerang DNA amplification (BDA); capture PCR (CPCR); inverse PCR (IPCR); novel *Alu*-PCR; panhandle PCR; polymerase chain reaction (PCR); restriction-site PCR (RS-PCR); splinkerette-PCR; targeted gene-walking PCR; vectorette-PCR.

Introduction

Some viral DNAs and almost all transposable elements (mobile element or transposons) can integrate into host genome DNA by means of a random or site-directed integration mechanism. The identification of the integration sites is important not only for an understanding of the molecular mechanism of integration but also for identifying novel cellular genes that are involved in cell proliferation and differentiation. Cloning of viral gene integrants via genomic library construction and Southern hybridization selection is very laborious and time-consuming. The conventional procedure has been applied in cloning the integrants of hepatitis B virus (HBV) [1–7] and of woodchuck hepatitis virus (WHV) [8, 9]. Since most of the specimens are usually obtained in small amounts, application of the polymerase chain reaction (PCR) to yield clonable amounts of DNA has been recommended. PCR requires a pair of primers annealed to sites at each end of the target DNA template in order for amplification to proceed. This is not applicable to the junction site of integration discussed here, because only one side of the fragment sequence in the integrant is known. In the past 10 years, several strategies have been developed to amplify unknown DNA flanking sequences. Inverse (or inverted) PCR (IPCR), novel *Alu*-PCR and vectorette-PCR (modified as design splinkerette-PCR) are PCR-based techniques used to amplify and clone unknown neighboring DNA junction sequences of the integrants. Although still not yet applied to junction site studies, targeted gene-walking PCR, restriction-site PCR (RS-PCR), capture PCR (CPCR), panhandle PCR and boomerang DNA amplification (BDA) may offer other alternative strategies. This review is intended to outline, compare and contrast these techniques.

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IPCR

IPCR is a modification of the PCR technique that is used to amplify cellular DNA adjacent to an integrated sequence after its intramolecular circularization [10–13]. The basic principle of IPCR is shown in figure 1. This method begins with the digestion of genomic DNA from the target tissue or cell line with a restriction enzyme which does not have a cutting site in the integrant. In HBV, for example, *Hin*dIII is one of the enzymes which can be used. After Southern hybridization and size fractionation of the integrant-containing DNA fragment, subsequent intramolecular self-ligation of these DNA fragments generates a small monomeric circle. Within this circularized form of DNA, conventional PCR technique is applied to amplify the junction region by two primers in opposite directions on the known integrant sequences. Hence, in IPCR, the intramolecular circularization of template is a key step for amplification [10]. Application of IPCR technique to detect the integration sites of HBV [14], human T-lymphotrophic virus type I (HTLV-I) [15–18] and some transposable elements [19, 20] have been reported.

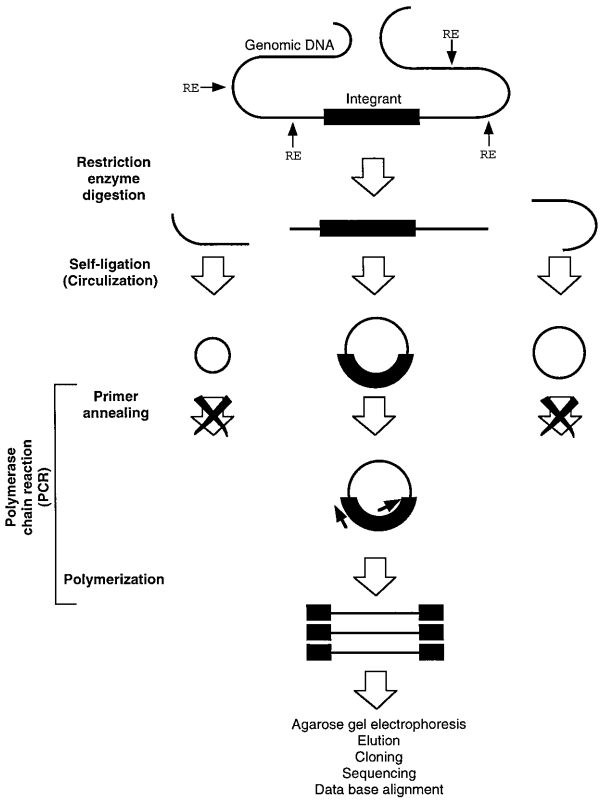

Figure 1. Principle of IPCR. The heavy bar and thin line represent the integrant fragment and cellular genomic sequence, respectively. In this particular example, the restriction enzyme cutting sites (REs) are not present in the integrant. The cross marks on arrows indicate that no primer annealing will occur in circularized DNA in contrast to circularized DNA with integrant. Primers for PCR are shown by arrows (see ref. 14 for detailed manipulations).

To avoid intermolecular ligation, the concentration of input DNA has to be decreased and results in a larger volume for ligation. Therefore, the circularization-ligation (self-ligation) step in IPCR is difficult to optimize. Frequently, no amplified DNA is obtained. This technique thus requires a relatively larger sample size to compensate for the low efficiency of ligation. Moreover, the noncircularized, free viral or transposon DNA fragment may interfere with the PCR reaction. These drawbacks make it difficult to study a foreign integrant DNA in small cancer.

Novel *Alu*-PCR

Alu element-mediated PCR (*Alu*-PCR) was first applied to amplify human genomes in a background of nonhuman genomes [21–23]. This restricted PCR is based on the fact that the *Alu*-repeated sequence is interspersed in the human genome at the mean interval of about 4 kb (for reviews see refs 24, 25). Hence, extending the applicability of *Alu*-PCR, the inserted foreign sequence can be directly amplified between the known inserted sequence and a human *Alu* consensus sequence and therefore identify the integrant-cellular junction sequence. Hence it is called 'novel *Alu*-PCR' [26]. The overall strategy is outlined in schematic form (fig. 2). Two specific primers are needed in *Alu*-PCR: one primer annealing to the known integrant sequence and the other to human *Alu* repeat sequences. In order to avoid illegitimate products which are amplified from *Alu* sequences themselves, two techniques have been suggested [26].

First, the primers should be synthesized by incorporating deoxyuridine triphosphates (dUTPs). This chemically modified primer can then be destroyed by uracil DNA glycosylase (UDG) after the first 10 cycles of amplification. Such modification can break the *Alu*-*Alu* specific amplification (see fig. 2, step b). Second, an asymmetric amplification is performed before UDG treatment (see fig. 2, step a). The primer on the known sequence is added at a 10-fold higher concentration than the primer for the *Alu* sequence [27]. In general, such a PCR reaction does not favor *Alu*-*Alu* amplification. Moreover, the design of the primer may also contain a tag sequence which can be applied to the other standard PCR protocols, such as nested or heminested PCR (see fig. 2, step c), to decrease the nonspecific amplification of PCR. A single primer control excluding the false-positive amplification and Southern hybridization have been suggested to facilitate cloning.

Novel *Alu*-PCR offers at least three advantages over IPCR. First, less DNA is required. Second, in contrast

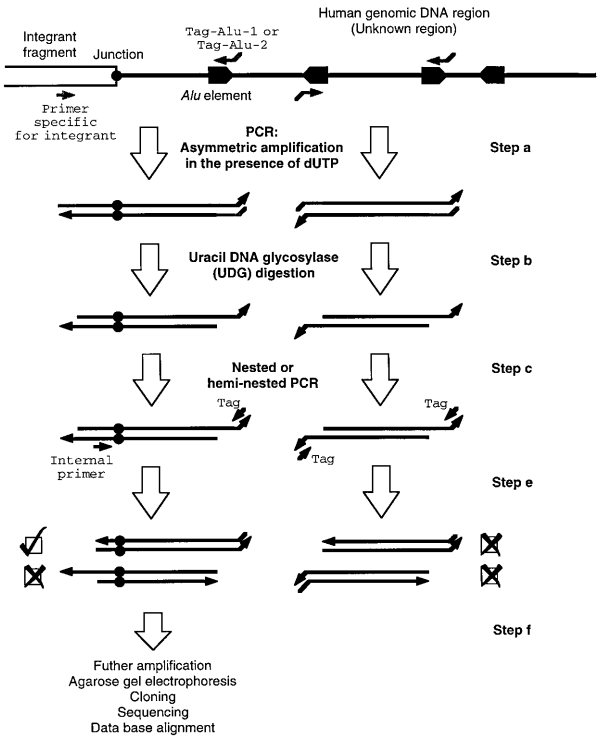

Figure 2. Principle of Alu-PCR. The open box and heavy line represent the integrant fragment and unknown human genomic sequence, respectively. The closed circle shows the junction site. Closed arrow boxes represent *Alu* elements on the human genome in different orientations. Gray arrows with tails represent the primer with tag sequence for the *Alu* consensus sequence, which is incorporated with dUTP. Primers for PCR are shown by small arrows. The cross and tick marks represent amplification. Only tick marks indicate the extension of the PCR reaction (see ref. 27 for detailed manipulations).

to IPCR, an intramolecular ligation reaction is not required in novel *Alu*-PCR. This can overcome the low efficiency of the self-ligation reaction. Third, novel *Alu*-PCR is based on only two steps, UDG digestion and conventional PCR procedures, substantially saving time. Using this protocol, some investigators have successfully identified cellular sequences flanking integrated human immunodeficiency virus-1 (HIV-1) [28, 29] and HBV [27] DNAs. Moreover, based on the same principle, the *Alu* primer can be replaced by any primer which can anneal to other genome repetitive sequences (for a review see ref. 30). The integration flanking region of human papilloma virus type 16 (HPV-16) has been identified by an HPV-specific primer and a human interspersed repetitive sequence (IRS)-specific primer [31]. However, this technique is not suitable for the case of an *Alu* repeat within a short distance. Unfortunately, many integrants are located adjacent to repetitive sequence [2, 6, 9, 32–35]. Furthermore, the use of novel *Alu*-PCR is also limited by the requirement for the adjacent repeat sequences to be in the correct orientation. In addition to the *Alu* sequence used in human samples, other species-specific interspersed repetitive sequences have also been reported (for review see refs 24, 30) and applied in the same approach [31].

Vectorette-PCR and splinkerette-PCR

Vectorette-PCR was first used for rapid isolation of terminal sequences from yeast artificial chromosome (YAC) clones [36], which is a method derived from cassette ligation-anchored PCR (LA-PCR) [37]. It is very similar to the principle of single-specific-primer PCR (SSP-PCR) [38], rapid amplification cDNA ends (RACEs) (for reviews see refs 39, 40) and rapid amplification genomic DNA ends (RAGEs) [41]. In these PCR methods, the ligated unit enables PCR to amplify the sequence interspersed between itself and a known primer, and avoid the requirement for circularization. The procedure of vectorette-PCR begins with the digestion of genomic DNA with a restriction enzyme to generate a 5′-overhang, and then follows by ligation of a linker, termed a vectorette unit, to the restriction enzyme-digested ends. The flanking sequences are then amplified using a known specific primer of the integrant and the universal vectorette-specific primer. The amplification strategy is shown in figure 3A. The vectorette unit contains a central noncomplementarity mismatched region resulting in a bubble shape as shown in figure 3B. Thus vectorette-PCR is also called bubble PCR.

However, undesirable amplifications of nonspecific 'end-repair priming' may involve the free cohesive ends of unligated free vectorettes and 5′-overhangs of unknown cellular regions. These ends are filled during the first cycle of PCR reaction. After the denaturing step, these ends are able to anneal together as shown in figure 3C. The complementary strand of vectorette primer can then be generated from unwanted fragments, which decreases the specificity of vectorette-PCR. The splinkerette was therefore designed as a hairpin structure on one strand rather than a central DNA mismatch [42], as presented in figure 3B. The advantage of splinkerette-PCR over vectorette-PCR is the elimination of the end-repair priming phenomenon. Some researchers have successfully identified flanking regions of transposons using this method [43].

Other options

Besides the techniques described above, other methods can also be used to clone the flanking sequence. Although the application of the following methods has not yet been routinely employed in finding sequences adjacent to the

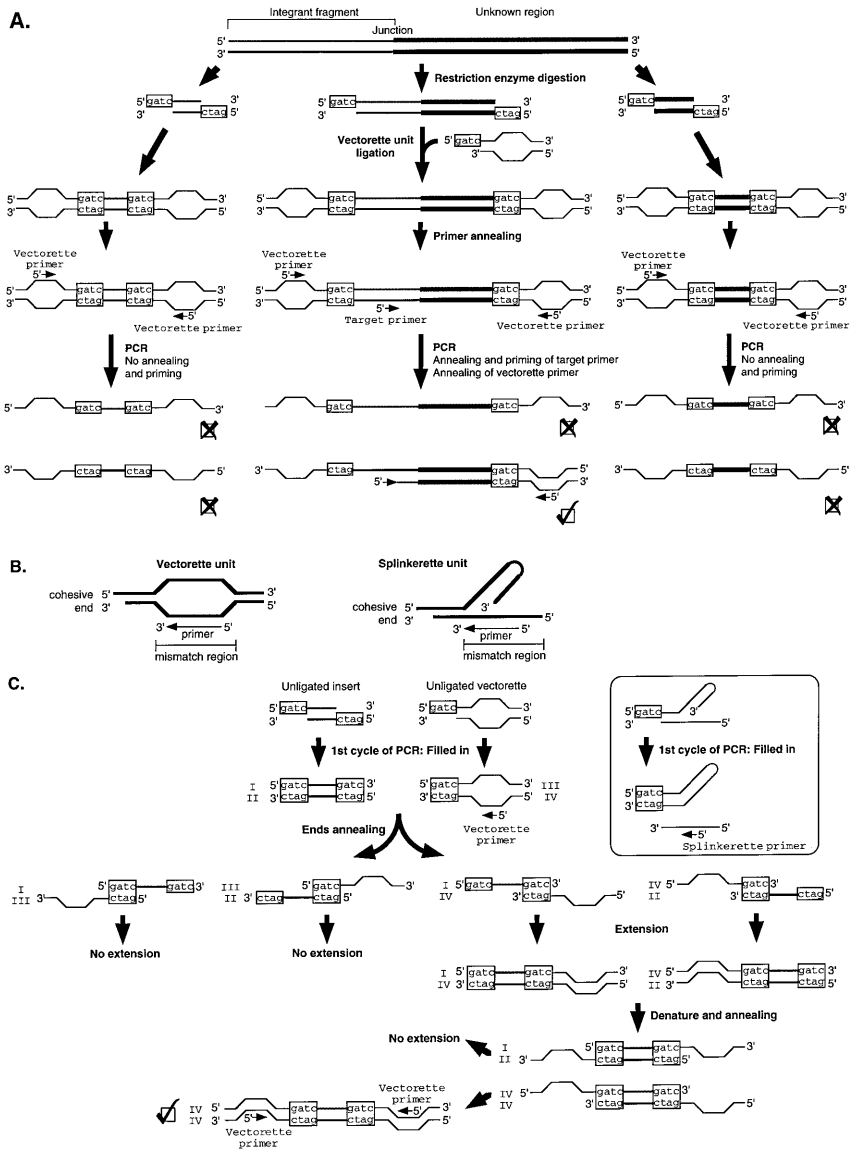

Figure 3. Principle of vectorette-PCR. (*A*) Two complementary strands of DNA are represented by gray and black lines. The heavy and thin lines represent the unknown genomic sequence and integrant fragment, respectively. A vectorette unit is shown in the figure. Primers are shown as arrows (see ref. 36 for detailed manipulations). (*B*) A schematic diagram comparing vectorette with splinkerette units. (*C*) A diagram showing the effect of 'end-repair priming'.

integrant, they are still recommended in seeking a junction region.

Targeted gene-walking PCR [44] has been used to amplify unknown DNA sequences adjacent to a known sequence by using the combination of a single targeted specific primer with nonspecific 'walking primers'. A schematic diagram of targeted gene-walking PCR is shown in figure 4. In this method, the first round of the PCR is carried out in different tubes containing a set of targeted primer and walking primer. The second reaction is an oligomer-extension assay involving the use of a [^{32}P]-labeled nested internal primer. In addition to increasing the sensitivity of the desired fragment, the isotope-labeled products can then be eluted from the dried gel and amplified in the following PCR procedure. Actually, the principle of this technique is similar to differential display PCR (DD-PCR) [45; for reviews see refs 46–48]. This method allows rapid detection without using a ligation or cloning procedure. Amplification occurs either upstream or downstream of a known

sequence. However, in order to increase the incidence of positive results, a series of walking primers has been designed, and at least 20 walking primers have been demonstrated [44]. In addition, a series of walking reactions are usually done in parallel. This can be laborious and time-consuming. Moreover, because of the mismatches, the 'walking primer' serves as an amplification primer. The stringency of the primer-template interaction is an important parameter [49]. Single primer control is essential to exclude false-positive results.

Another method, termed restriction-site PCR (RS-PCR) [50], is similar to targeted gene-walking PCR. In the first reaction of RS-PCR, the set of 'walking primers' is replaced by a set of restriction site oligonucleotides (RSOs) designed for a given RNA polymerase promoter sequence with a core anchor region and restriction enzyme cutting site as shown in figure 5A. The second reaction also uses PCR of internal primers and the same RSOs. A schematic diagram of RS-PCR is shown in figure 5B. After PCR amplification, an appropriate RNA polymerase can specifically transcribe the ROS-containing products and reverse transcription (RT) can convert the RNA product into a DNA template for sequencing. Since RS-PCR is similar to walking PCR in principle, they share the same advantages and limitations.

Capture PCR (CPCR) is based on enrichment of interested sequences by a streptavidin-coated support for the PCR reaction [51]. After initial ligation of oligonucleotide linkers to all restriction ends, the biotinylated specific primers to known sequences are used for an extension reaction. These biotin-labeled extension products are immobilized on streptavidin-coated paramagnetic beads and then used as templates in a PCR reaction. The principle of this approach is shown in figure 6. This method is simple and highly specific. Moreover, no cloning procedure is required if solid-phase sequencing in used.

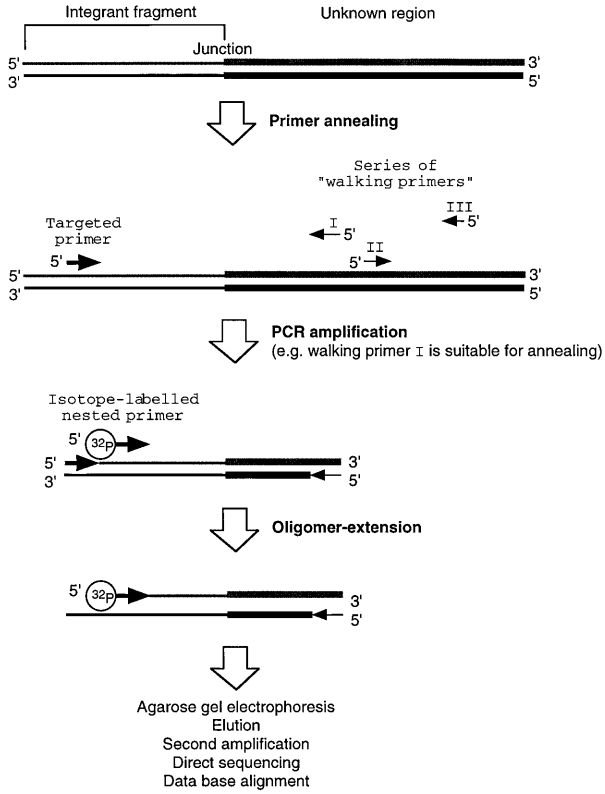

Figure 4. Principle of targeted gene-walking PCR. Two complementary strands of DNA are represented by gray and black lines. The unknown region of genomic DNA and known integrant sequence are shown as heavy and thin lines, respectively. Primers for PCR are shown with arrows (see ref. 44 for detailed manipulations).

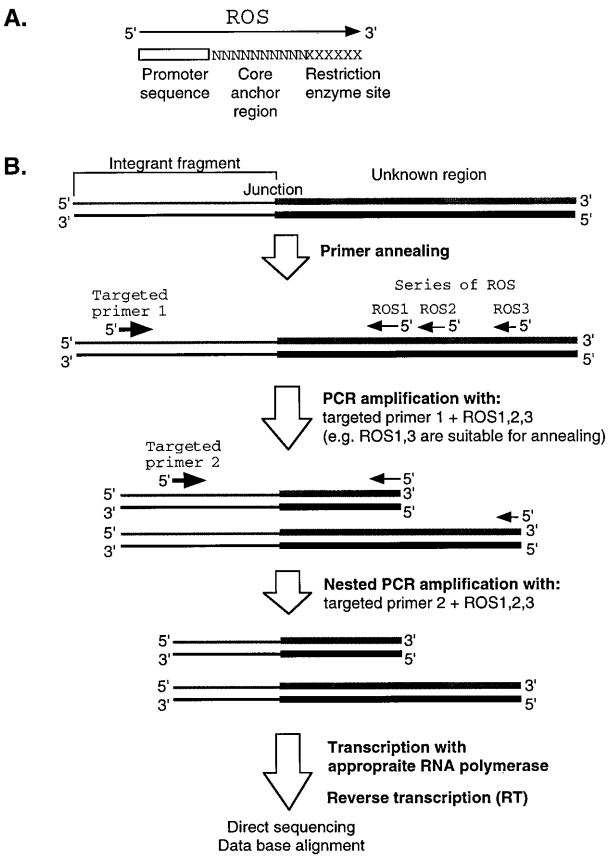

Figure 5. Principle of RS-PCR. (*A*) Structure of an RSO. (*B*) Schematic representation of the strategy of RS-PCR. Two complementary strands of DNA are represented by gray and black lines. The unknown region of genomic DNA and known integrant sequence are shown as heavy and thin lines, respectively. Primers for PCR are shown with arrows (see ref. 50 for detailed manipulations).

Panhandle PCR has been applied to clone unknown DNA flanked by a known sequence [52, 53]. This method has been termed 'panhandle PCR', because it generates a template shaped like a pan with handle. The principle is illustrated in figure 7. First, the prepared human genomic DNA is digested with a restriction enzyme to yield a 5′-overhang, and subsequent dephosphorylation of the DNA fragment by alkaline phosphatase prevents the self-ligation of genomic DNA fragments. Subsequently, the treated genomic DNAs are then ligated to a phosphorylated single-stranded oligonucleotide. This single-stranded oligonucleotide has two features: its 5′-end is complementary to the single-stranded ends of restriction enzyme-digested genomic DNA, and its sequence is homologous with a part of the integrant fragment. After denaturation, similar to IPCR, intrastrand annealing is performed due to the sequence homology between ligated oligonucleotide and internal sequence of integrant. After the extension of the recessed 3′-end using DNA polymerase, the pan-

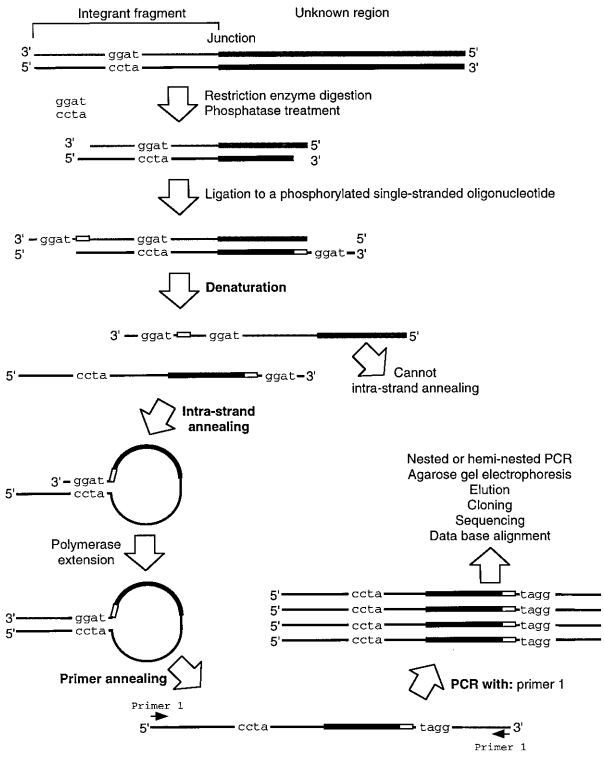

Figure 7. Principle of panhandle PCR. Two complementary strands of DNA are represented by gray and black lines. The unknown region of genomic DNA and known integrant sequence are shown as heavy and thin lines, respectively. The letters on thin lines indicate the specific annealing region for the internal portion of integrant and ligated oligonucleotides. The PCR primers are shown as arrows (see ref. 52 for detailed manipulations).

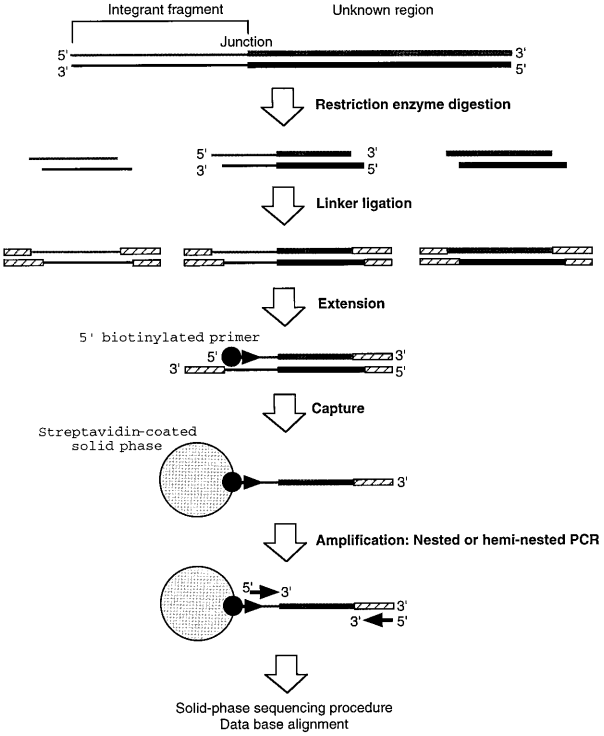

Figure 6. Principle of capture PCR. Two complementary strands of DNA are represented by gray and black lines. The unknown region of genomic DNA and known integrant sequence are shown as heavy and thin lines, respectively. The linker for ligation is shown with hatched boxes. Biotinylated primer for extension is shown with an arrow having a closed circle. Primers for nested or heminested PCR amplification are shown by arrows (see ref. 51 for detailed manipulations).

handle structure is formed, which contains an inverted repeat of the integrant. Conventional PCR is used with two specific primers in order to amplify the junction fragment; panhandle PCR has been modified so that only a single primer is necessary [54]. Because of the similarity of panhandle PCR to IPCR, the limitations are also comparable. Note that the efficiency of intrastrand annealing is an important step.

Another method, called boomerang DNA amplification (BDA), is similar to vectorette-PCR in the first step of adding an adapter but involves only a single primer that is extended from a single primer binding site and then is looped around the other strand [55]. The resulting strand has an inverted repeat and the amplification principle is similar to panhandle PCR. However, practical application of BDA has not yet been tested.

Overview

There is much interest in the cloning of unknown neighboring DNA from a presumed integration site. All

Table 1. Cloning unknown neighboring DNA techniques.

Method	DNA digestion	Ligation	Amount of DNA used	Steps to optimize	Nonspecific products (false positive)	Sensitivity
Inverse PCR (IPCR) (fig. 1)	yes	self-ligation (intramolecular circularization)	D: 2–5 μg L: 0.25–5 ng/μl	1. requires large amount of DNA sample 2. DNA concentration of self-ligation	from free non-integrated DNA and nonligated DNA	low
Novel *Alu*-PCR (fig. 2)	no	no	P: 20–106 ng	1. *Alu* orientation consideration 2. species-specific repetitive sequence	from *Alu-Alu* amplification	high
Vectorette- or splinkerette-PCR (fig. 3A)	yes	vectorette unit ligation	D: 1–2 μg L: 1–2 μg	1. ligation condition 2. laborious and time-consuming	from 'end-repair priming' (fig. 3C).	medium
Targeted gene-walking PCR (fig. 4)	no	no	P: 50 pg–15 ng	primer set design	from single walking primer amplification	high
Restriction-site PCR (RS-PCR) (fig. 5B)	no	no	P: 200 ng	RSO set design	from single RSO primer amplification	high
Capture PCR (CPCR) (fig. 6)	yes	linker ligation	L: 100 ng	ligation efficiency	from single biotinylated primer	medium
Panhandle PCR (fig. 7)	yes	single-stranded oligonucleotide ligation	D: 1.2–5 μg L: 0.5–2.5 μg	1. ligation efficiency 2. intrastrand looping condition	from single target primer	medium

D, DNA digestion reaction; L, ligation reaction; P, PCR reaction.

methods described are modifications of PCR and are compared in table 1. The PCR technique is used in amplifying trace amounts of starting template DNA [49, 56, 57]. However, one limitation is the need for the sequence of two target-specific primers that flank the region which is intended for amplification. The problem here is how to allow the direct amplification of DNA without a prior knowledge of sequence information. This is accomplished by using many different methods with a fundamental understanding of the principles of each method. This review provides techniques for cloning viral or transposon integration sites. The techniques can also be applied to the determination of YAC endpoints, genomic breakpoints (deletion or translocation), intron-exon junctions and sequences adjacent to regulatory regions. Among all the techniques introduced above, novel *Alu*-PCR and targeted gene-walking PCR are highly recommended in isolating integrants. These methods are highly sensitive. They use a straightforward conventional PCR protocol and require neither genomic DNA digestion nor ligation reactions. Although false-positive result may occur, the single-primer control plus Southern hybridization help to minimize the problem.

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