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Cloning Strategies Using a Third Irrelevant Enzyme Site (TIES) to Overcome Certain Cloning Problems

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ABSTRACT

We describe a cloning strategy, third irrelevant enzyme site (TIES), based on three-component assembly ligation reactions to overcome seven types of problematical cloning reactions. Implementation of the TIES strategy has made it possible to obtain the desired recombinant where the corresponding approach using conventional recombinant DNA methodologies and two-component ligations failed to give the desire product.

INTRODUCTION

Molecular cloning, the generation of specific recombinant DNA molecules, is a basic technology in molecular biology. Some clonings may be difficult to accomplish because of the absence of appropriate or workable restriction sites, from contiguous, overlapping or multiplicity of interfering restriction sites and from problems with generating appropriate directional insertion. It becomes worthwhile to find basic ways to improve cloning reactions so that the desired recombinant is more easily obtained, thus saving time and effort expended on the screening end. This is certainly the case when problems cause the efficiency of recombination to be too low to easily screen for recombinants without resorting to massive screening protocols based on hybridization (6) or PCR-based methods

(7). Here a new strategy is described for certain problematic DNA clonings. The principle is based on using a third irrelevant enzyme to generate an adaptor DNA fragment that is combined with a three-component assembly-type ligation reaction to produce the desired recombinant. The strategy named the third irrelevant enzyme site (TIES) involves the isolation of an essential fragment of DNA from the vector or insert that is re-added to serve as a specific adaptor to *tie* together the assembly of the desired recombinant in a three-component DNA ligation reaction. In the theory of the two-component ligation for DNA recombination (5), the concentrations of the reactants and, in particular, their termini, are very crucial. Either more self-ligation, e.g., linear concatenation, or oligomerization of insert (i) or vector (v) is favored over recircularization of these components (or vice versa depending on the termini concentration values, i/j), which has important ramifications for obtaining the desired v-i recombinant. A recent application of this for blunt-end ligations is the development of two-stage ligation reactions to obtain higher v-i recombinant yields (3,8) or a polymerase chain reaction (PCR) salvage methodology (11). In a three-component ligation reaction, the fragments can be designed to join in a desired orientation if an assembly order is imposed by judicious choice of incompatible cohesive termini in the reaction. Although the absolute yield of recombinant ligation among all the ligation products in this type of ligation reaction is lower since the assembly is designed to be made specific, theoretically only the desired recombinants by design will form viable clones, while dimer, linear concatenates from oligomer of insert or vector and their recircularized products cannot form viable recombinants after transformation. In practice, almost all of the transformed colonies yield the desired recombinant, and the overall efficiency of cloning is high because the yield of false positives is constrained. The net effect of TIES is to make certain gene recombinations possible and easier than that which would be otherwise problematical or impossible by conventional two-component DNA cloning reactions.

MATERIALS AND METHODS

The restriction endonucleases were obtained from either New England Biolabs (NEB) (Beverly, MA, USA), Promega (Madison, WI, USA) Life Technologies (Gaithersburg, MD, USA); T4 DNA ligase and DNA polymerase I large fragment (Klenow) were from

Table 1. Comparison of Recombinant Yields for Problem Clonings by TIES Method vs. Conventional Two-Component Reaction

Cloning Problems	TIES	Conventional Method
1. Filipping Insertional orientation	7/8 positive, more than than 1000 colonies. 60%–100% positive*	Cannot be done if vector has no homolog with opposite orientation cloning site.
2. Cloning between too short a distance or overlapping sites:		
A. Overlapping sites.	9/10 positive, 800 colonies. 50%–100% positive.	Cannot be done.
B. Too short a distance.	6/8 positive, more than 500 colonies, efficiency same as 2A.	Very low efficiency, most are self-ligation colonies, usually less than 1% positive.
3. Multiple interfering sites:		
A. More than one interfering site.	7/11 positive, about 1500 colonies, more than 50% positive.	Cannot be done.
B. One interfering site.	5/8 positive, 150 colonies. Highly efficient, efficiency same as 3A. No false positives from problems with controlling the digestion.	Use partial digestion strategy, low efficiency, most false positives are from self-ligation or from complete digestion.
4. Forced directional cloning into a single site.	4/8 positive, 800 colonies, 30%–80% positive.	Cannot be done.
5. Transferring fragments from different vectors.	3/8 positive, about 700 colonies. 40%–100% positive.	Cannot be done.
6. Single-site cloning.	2/8 positive, 200 colonies. Without dephosphorylation, highly efficient, more than 20% positive.	0/40 positive, 2000 colonies. Low efficiency, with high concentration of inserts, can get concatenated inserts.
7. Block interfering site.	Sequential digestion and ligation, 4/10 positive, 200 colonies.	Not applicable.

*The proportion of positive recombinants per total number of screened colonies and the total number of colonies per transformation. Percent positive indicates range of positive recombinants from several experiments.

NEB, mung bean nuclease (Boehringer Mannheim, Indianapolis, IN, USA) and RNase A Type I-AS from Sigma Chemical (St. Louis, MO, USA).

For the basic TIES subcloning method, the vector is divided into two aliquots: one aliquot is cut with one of the two cloning site restriction enzymes and with an irrelevant enzyme at a third site, while the second aliquot is cut with the other cloning site restriction enzyme and at the same third irrelevant site. The corresponding vector backbone and the vector adaptor fragment thus generated are separated and eluted from an agarose gel (see strategies under different subheadings for specific details about which vector fragments should be selectively recovered in the different applications). The three-component ligation reaction is assembled from the vector backbone (0.5–1.0 µg), the vector adaptor (or foreign adaptor in the strategies depicted in Diagrams 3B and 5) and the foreign fragment to be cloned at molar stoichiometric ratios of 1:2–3:2–3, respectively (for the strategy depicted in Diagram 6, the ratio is 1:1:2–3, while for the strategy depicted in Diagram 7, use 10 µg in the first digestion and ligation step, and then in the second step, it is the same as a conventional two-component reaction). The same amount of vector backbone is used for a self-ligation control reaction and gives an estimate of false-positive yield. The ligation reaction is carried out at 16°C overnight in 20 µL with 1 µL of 6 Weiss U/µL of T4 DNA ligase (for ligation reaction involving blunt ends, 0.5 µL of 30 U/µL ligase is recommended) in 50 mM Tris-HCl (pH 7.8), 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, 50 µg/µL bovine serum albumin after which half of the ligation mixture (about 250–500 ng) and 100 µL of XL1-Blue or JM109 (Stratagene, La Jolla, CA, USA) competent cells are used for transformation. Recombinant clones are generated and analyzed by standard procedures described in (References 1 and 10) for isolation, restriction mapping and DNA sequence analysis.

The criteria for selection of the third irrelevant enzyme site are as follows: *(i)* the enzyme should not be an

isoschizomer or restriction enzyme yielding compatible cohesive termini with either of the two cloning enzymes; *(ii)* an enzyme that generates a site with cohesive termini is better than one with blunt termini; *(iii)* the choice of enzyme depends on the location of available unique restriction sites, but it should be chosen so that the vector can be cut into two fragments with resolvable differences in size for ease of separation and recovery from the gel; and *(iv)* it does not matter that this site is located on the left- or right-hand side of the two cloning sites, but when a left-hand side third site is selected, a corresponding change in fragment recovery should be made (the diagrams are for a right-hand situation, but the problem is symmetrical; see the strategy depicted in Diagram 1 for more details). Last, it is important to recover the correct pair of fragments from the gel, because the gel banding pattern is very similar after electrophoresis (except in the strategy depicted in Diagram 3), and different combinations of fragments will give opposite insertional orientations.

RESULTS

Specific types of clonings where the TIES approach has yielded the only results or better results than the conventional two-component recombinant reaction are described under the following numbered subheadings that also correspond to the numbered diagrams in Figure 1. In Table 1, a comparison of the yield of recombinants by the TIES method vs. the standard two-component reaction is tabulated in the same order. These examples may not be exhaustive of where the TIES approach may be appropriate for overcoming cloning problems, but they are the only ones encountered so far where TIES offered a more efficient solution for obtaining a desired recombinant. Due to space limitations, data on the analysis of specific types of TIES clonings under each subheading are available upon request with detailed protocols for the reaction.

1. **Flipping a sequence to obtain recombinants in the reverse orientation**. Sometimes two cloning sites, A and B in a vector, are in the reverse ori-

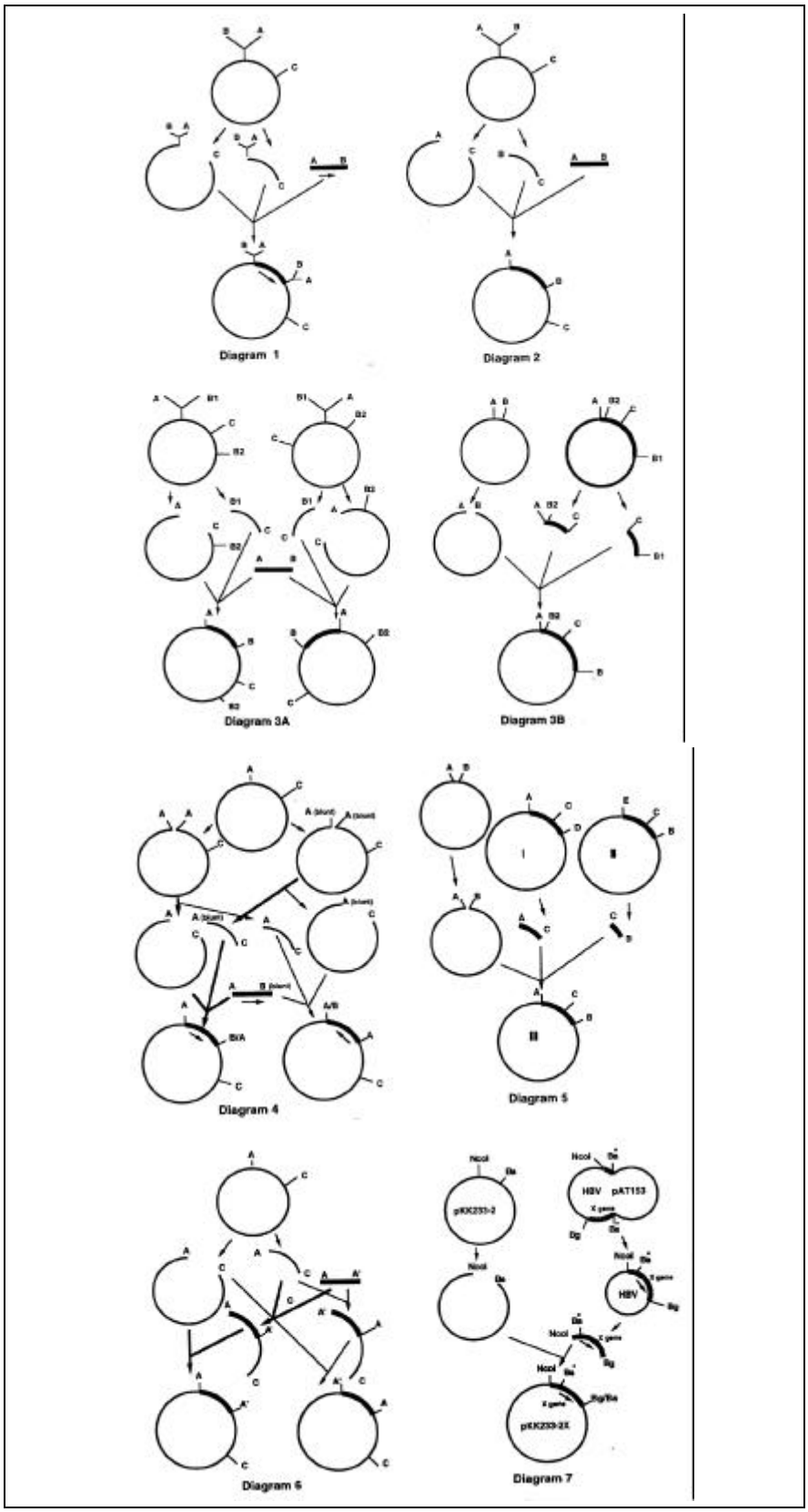

Figure 1. TIES strategies. Diagrams 1–7 illustrate the different TIES strategies used to overcome some common cloning problems. Each of these strategies is described in the corresponding subheading under Results. Ba: *Bam*HI, Bg:Bg*l*II.

entation of what is required for the insertion of a recombinant. By selecting a third irrelevant restriction site C, this problem is easily surmounted as shown in Diagram 1. The vector is digested in two aliquots with A and C, and B and C, respectively. The A(B)^C vector backbone and B(A)^C adaptor are recovered [C site can also be on the left-hand side, in this case the A(B)^C vector adaptor and B(A)^C vector backbone should be recovered, otherwise it will form a reverse insertional orientation] and added to the foreign fragment A-B that is desired to be cloned. These are assembled into a TIES simultaneous three-fragment ligation with a stoichiometric ratio described in Materials and Methods. Note that the fragment is inserted in the direction A(5′)→B(3′) rather than B(5′)→A(3′), the original orientation in the vector. This strategy was applied in the construction of pMS, where the *Hin*dIII and *Sma*I sites were in the reverse orientation in the pMG3 vector with respect to the preferred orientation for the cloning of the SV40 early promoter and enhancer cassette.

2. **Cloning between too short a distance or overlapping sites.** When two restriction sites A and B are overlapping, contiguous or separated by a very short intervening stretch of sequence <10 bp, it can be difficult to get a complete digestion of the vector at one or the other restriction site. This in turn creates a problem for setting up an efficient two-component cloning reaction. Furthermore, the ratio of self-ligation product can be very high causing screening problems. Diagram 2 shows that cloning is made easier by relying on a third irrelevant site C. Two aliquots of the vector are digested with A and C, and B and C restriction enzymes, respectively; then the A^C vector backbone and the B^C vector adaptor are recovered and added to fragment A-B, which is the desired insert or foreign DNA to be subcloned in a three-component ligation reaction. For example, when a foreign fragment having *Xba*I and *Eco*RI termini is to be inserted at overlapping *Xba*l and *Eco*RI sites (TCTA[GA)ATTC] within baculovirus vector pVL1393 (Pharmingen, San Diego CA, USA) containing such a multiple cloning site, no other strategy but this can be used for cloning.

3. **Multiple interfering restriction sites in the vector or insert.** When the subcloning site is not a unique site in the vector, partial digestion and shotgun cloning are usually used to get the desired recombinant. Generally this leads to massive screenings with no guarantee that the desired recombinant will be present. If there is just a single interfering site, it is possible to mutate it; however, more often than not, this approach is not feasible if there is more than one interfering site or a site must be retained because it is in a special codon configuration. Diagram 3A shows the alternative TIES procedure. For identification, two B sites are

marked as B1, B2 (B2 can be one or more, and there are two possibilities for the position of site A, which can be located in between the B sites or on one side of the B sites). Suppose the fragment is going to be cloned between A and B1. The vector is digested with A and C, and the A^C vector backbone (including B2 site) is recovered; then the original vector is cleaved by C and B, and C^B1 fragment (there must be no other B site between B1-C) is recovered. These two fragments and the foreign one are assembled in a three-component simultaneous ligation as before. In the case where the foreign fragment has multiple B sites, switch what is vector and insert as shown in Diagram 3B (the problem is symmetrical). In the course of construction of pIRES-FLP, this tactic was used to overcome the four *Hin*dIII sites in pOG44 (Stratagene).

4. **Forced directional cloning into a single-site.** When there is only a unique restriction site or a single compatible cohesive terminus between the vector cloning site and one of the two ends of the foreign fragment, the fragment can still be cloned into the vector in a definite orientation with TIES. Diagram 4 shows how to control the resultant orientation of the insert. The vector is digested with A, then split into two aliquots, one of which is blunted by the Klenow or mung bean enzyme, and the other one is left untreated. The B site of the foreign fragment is also blunted. By recombining selectively the A(not blunt)^C vector backbone and the C^A(blunt) adaptor of the vector with the A-B(blunt) insert, the insertion is controlled to give A(5′)→B(3′) direction. On the other hand, by recombining selectively the A(Blunt)^C vector backbone and C^A(not blunt) adaptor of the vector with the A-B(Blunt) insert, the insertion goes in B(5′)→B(3′)direction.

5. **Transferring fragments from different vectors having at least one common site.** When an insert of interest has been cloned into more than one vector (e.g., I and II), but needs to be transferred into a new vector (III), it can be force-ligated into the new vector III by relying on a third irrelevant site in the fragment to obtain different parts of the insert from vectors I and II. Diagram 5 shows that by cutting the fragment with A and C in vector I and with C and B in vector II, the A^C/C^B fragments equivalent to the whole A^B insert can be cloned into a new vector by a forced ligation reaction with a high yield of recombinants. This strategy is often used for generating suitable restriction enzyme termini from the vector other than using linkers. Another application of this strategy is when the vector and insert sizes are similar, by cutting the insert (or vector) at a third irrelevant site into two fragments, the problem of size interference in gel recovery and subsequent cloning is eliminated. When NS–β-gal from pJK2 (3.4 kb) was subcloned into pBluescript® [NS is nuclear localization signal] from pJK2 (with identical vector size of 3.4 kb), NS-β-gal was cut into two *Hin*dIII-*Eco*RI and *Eco*RI-*Bam*HI fragments and thus easily resolved from vector that on reassembly in a TIES reaction gave the desired recombinant.

6. **Sequential three-fragment ligation reactions for a single-site cloning.** Self-ligation is the greatest problem in a single-site or blunt-end cloning if the vector ends are not dephosphorylated (12), but if they are, the efficiency can sometimes be extremely low. This can be remedied by methods employing two-stage ligation reactions (3,8) or a salvage PCR (11). However, by choosing a third irrelevant restriction site in the vector and then ligating the fragments in two separate reactions, a low efficiency single-site or blunt-end ligation can be changed into a forced ligation, thereby increasing the yield of recombinants. Diagram 6 shows this strategy. The vector is digested with A (cohesive or blunt termini) and C, and the A^C backbone and C^A adaptor of the vector are recovered; then the foreign fragment (indicated with A, A′ to identify the direction) and C^A adaptor fragment are ligated for 2 h at 16°C in 15 µL. (The molecular ratio is 2–3:1 in order to block most of the A site of the C^A fragment.) In the second stage, the A^C vector backbone is added to this ligation mixture, and the ligation is continued at 16°C overnight by adding another aliquot of T4 DNA ligase and adjusting the volume to 20 µL. (The overall molecular stoichiometric ratios are 1:2–3:1 for vector backbone, foreign fragment and vector adaptor.) After transformation, it is easy to screen for recombinant colonies in both orientations. While making a construct of pXT1-RX, this approach was used to obtain positive colonies without vector dephosphorylation, whereas using the conventional two-component ligation method, no positive colonies were obtained (Table 1).

7. **Blocking an extra site before cloning.** Sometimes there is a need to block an extra restriction site during cloning. This happens when the DNA is subcloned into a vector in a single site, and the desired gene is split in the cloning site. The DNA can be first cut out with the cloning enzyme and cycled by self-ligation; then a whole copy of the gene can be excised from the concatenated DNA. This reaction, shown in Diagram 7, is also a kind of sequential ligation. In the course of the construction of pKK233-2X, when the *Nco*I-*Bam*HI and *Bam* HI-*Bgl*II fragments of the human hepatitis B virus X gene were recovered and used in the ligation reaction, the following problems were encountered: First, the recovery yield of the *Nco*I-*Bam*HI fragment was very low because of its size (28 bp). Second, *Bam*HI and *Bgl*II had compatible ends, which led to two uncontrolled orientations of insertions (*Nco*I→*Bam*HI→*Bgl*II and *Nco*I → *Bgl*II→*Bam*HI), for which only the former is desired. Third, in the course of ligation, the *Bam*HI-*Bgl*II fragment was easily lost. By first cycling the *Bam*HI-*Bam*HI of HBV genomic sequence in a self-ligation reaction, all the difficulties were overcome in subsequent steps because the *Bam*HI site became blocked. Then the whole X gene was obtained by cutting with *Nco*I and *Bgl*II.

DISCUSSION

A strategy utilizing a third irrelevant restriction site which generates an adaptor DNA fragment and which employs a three-fragment assembly ligation reaction either in sequential order or in concerted reaction depending on the application offers a very important methodological tool for obtaining the desired recombinant DNA products. By

using the TIES strategy, we have documented how to overcome the many difficulties found in conventional two-component recombinant DNA reactions. Due to the more complicated ligation dynamics in a three-component reaction, the only requirement is to use a larger amount (0.5–1 μg) of reactant DNA molecules compared with a two-component ligation reaction. On the other hand, the rate of false positives is lower and often 0% of those recombinants formed, which ensures an easy time in obtaining the desired recombinant and makes the requirement of more DNA a moot inconvenience. In theory, the TIES strategy can give 100% positive recombinants (except in the strategy depicted in Diagram 6 where it is impossible to block entirely the A site of the vector adaptor, but adding more foreign fragment to the ligation mixture may help by increasing the blocking efficiency at the cost of creating multiple inserts). Usually the false positives are due to contamination from incomplete digestion of the cloning vector backbone or contamination of the vector backbone with the foreign fragment during elution and recovery from the gel. Sometimes a small proportion of false-positive colonies arises because of the plasmid recombination in the host cells after transformation, a problem also encountered in the conventional two-component clonings. We have highlighted seven different applications where the TIES strategy rectified problem cloning reactions and simplified obtaining the desired recombinants. In fact, this approach has proven to be very useful with more than 30 different problem clonings successfully overcome by using different TIES strategies with recombinant insert sizes ranging from 150 to 3.4 kb and vector sizes up to 10 kb (pXT1; Stratagene). Recently, expert programs incorporating artificial intelligence algorithms have been developed to pick optimized cloning strategies for a given cloning problem (2,4,9). However, such programs utilize a limited repertoire of strategies, which are not designed to address the cloning problems for which we describe solutions using the TIES strategy. By considering this strategy as an alternative to problem two-component DNA recombinant reactions as described under Results, certain cloning problems can be readily approached and have a straightforward solution.

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Cloning of Size-Selected Human Immunoglobulin Heavy-Chain Rearrangements from Third Complementarity-Determining Region Fingerprint Profiles

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ABSTRACT

Methods have been developed to rapidly visualize the size distribution of third complementarity-determining regions (CDR3) in immunoglobulin (Ig) and T-cell receptor (TCR) molecules. DNA fragments spanning the Ig or TCR CDR3 are generated by PCR using primers at fixed positions in the variable and constant segments. These fragments differ in length due to size variation of the CDR3s. Visualization of the amplification products in polyacrylamide gels as a "CDR3 fingerprint profile" is a rough measure for the complexity of the Ig and TCR antigen-binding specificities. We report an adaptation of this method for the analysis of human Ig heavy-chain genes that incorporates silver staining, which allows for the fine analysis of specific regions of the profiles. This is especially useful for the study of low-abundant transcripts.

INTRODUCTION

Several studies have recently described a method of third complementarity-determining region (CDR3) "fingerprinting" or "spectratyping" that