REFERENCES


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Wrapper Arms Strategy to Construct Multicomponent Plasmids Using Three-Piece Ligation

The introduction of DNA fragments into living cells is a basic molecular technique that allows in vivo investigations into the functions of specific DNA fragments or the direct manipulation of the host genome as used in the creation of knockout mice (1). Typically, multiple DNA components that are to be introduced into cells are assembled using existing prototypic plasmids. As an example, a cDNA fragment is inserted into a plasmid that has a promoter and a polyadenylation signal to make an expression plasmid (4). It is expected that the prototypic plasmids will be easy to manipulate to construct the desired plasmid and then easy to modify to meet the investigator’s needs. However, as the numbers of DNA components increase, these multicomponent plasmids become extremely hard to modify, primarily because they lack the appropriate restriction enzyme sites.

To overcome this difficulty, we introduce the wrapper arms strategy, which is applicable to the construction of a variety of plasmids. We also present prototypic plasmids designed using this strategy to make expression plasmids. Many commercially available plasmids can be used in combination with our plasmids to yield expression plasmids with different promoters, different drug resistance genes or different epitope tag sequences at the end of the cDNA.

The wrapper arms strategy uses the three-piece ligation (3PL) protocol to assemble the final plasmid from the separate plasmids. Here, we use the 3PL protocol to make circular DNA molecules from three DNA fragments that have three different pairs of cohesive ends in a permuted order (A-B, B-C and C-A) (Figure 1a). In the wrapper arms strategy, the left arm plasmid (LAP) provides the 5’ end of the final fragment, while the right arm plasmid (RAP) provides the 3’ end of the final fragment. The middle segment is derived from a fragment excised from another plasmid or from a fragment generated by PCR. In our example, pCMVLA1 (LAP) provides the 5’ end of the final fragment (promoter), while ptkneoRA1 (RAP) provides the 3’ end of the final fragment (drug resistance gene) (Figure 1a). It is important to use a restriction enzyme that cuts the ampicillin resistance gene (Amp') as enzyme A. Fragments A-B, B-C and C-A shown in Figure 1a are purified by agarose gel electrophoresis after digestion with the appropriate restriction enzymes, ligated by 3PL in a 20-µL volume for 1 h, introduced into E. coli and selected by ampi-

Figure 1. Sample of a plasmid system designed for the wrapper arms strategy. (a) Fragment A-B in pCMVLA1 contains the polyadenylation signal and the CMV promoter. Fragment C-A in ptkneoRA1 has a splicing sequence, a polyadenylation signal and a transcription unit containing a drug resistance gene. Enzymes B and C are chosen from those sites available in the polylinker sequence. These two fragments are ligated together with a cDNA to form the final construct. (b) Graphical representation of constructs used for transfection and the luciferase activity in cells treated with varying concentrations of Zn. pMT-luc-tkneo has the sheep MT-1a promoter (3). Relative luciferase units (RLUs) were calculated by dividing measured luciferase activity by µg protein in the cell lysate.

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cillin to isolate the final construct.

In the wrapper arms strategy, the number of unwanted constructs is low because (i) each fragment is agarose-purified and (ii) enzyme A (Figure 1a) cuts the Amp<sup>+</sup> gene, only allowing colonies with the reconstructed Amp<sup>+</sup> gene to grow. For enzyme A, AhdI, Asel, BglII, BpmI, BsaI, BsaHI and PvuI are examples of enzymes that can be used. The number of correctly ligated circular DNA molecules is expected to be low in the 3PL; therefore, competent cells with high transformation efficiency are required. For chemical transformation, XL1-Blue MRF<sup>+</sup> cells (Stratagene, La Jolla, CA, USA) have been used with success, and, for electrotransformation, DH10B™ cells (Life Technologies, Rockville, MD, USA) have been used. When properly performed, more than 70% of the colonies contain the expected construct.

The LAPs and RAPs have simpler structures than the final constructs and are easier to modify individually. In addition, many commercially available plasmids have structures in common with either LAP or RAP and can be used interchangeably. For our plasmid system (pCMVLA1 and ptkneoRA1), pLND (Invitrogen, Carlsbad, CA, USA), pLXSII and pBI (Clontech Laboratories, Palo Alto, CA, USA) can be used as LAPs, while pTracer<sup>TM</sup>-CMV2, pcDNA3/V5-HisA (Invitrogen) and pRES-neo (Clontech Laboratories) can be used as RAPs. By using combinations of these plasmids, a variety of features can be introduced such as inducible promoters (pLND and pBI), transfection markers (pTracer-CMV2), epitope tags (pcDNA3.1/V5-HisA) or an internal ribosomal binding site (pRESneo). The ease of modification is one of the advantages of the wrapper arms strategy.

In a typical plasmid construction, fragments are inserted into a plasmid one after another. This procedure requires enzyme sites unique to both the fragments and the plasmids. As more components are integrated into a plasmid, the number of unique enzyme sites decreases rapidly, making further plasmid manipulation much more difficult. In the wrapper arms strategy, as long as three fragments (fragment A-B, B-C and C-A in Figure 1a) can be isolated individually by agarose gel electrophoresis, enzymes A, B and C do not need to be unique in the LAP, the RAP, the middle segment and the final construct. This provides the advantage of more restriction enzyme choices for the plasmid construction.

Another advantage of preparing LAPs and RAPs for the wrapper arms strategy is that they can yield several useful plasmids as side products. In our sample plasmid system, ligation of only the LAP and the RAP produces an expression plasmid with a polylinker suitable to insert cDNAs (Figure 1a). Ligation of only the RAP and the cDNA produces a plasmid with a polylinker suitable to test different kinds of promoters.

To confirm that the plasmids constructed by the wrapper arms strategy work as expected, we transfected two constructs into BEAS-2B cells, an Adenovirus SV40 Tag immortalized human bronchial epithelial cell line (Figure 1b) (2). The neo-resistant colonies expressed luciferase constitutively (cytomegalovirus or CMV) or an inducible manner (metallothionein 1a or MT-1a), demonstrating that the plasmids worked as expected.

When designing new plasmids suitable for the wrapper arms strategy, it is important to consider what DNA fragments are contained in both the LAP and RAP. The middle segment is the easiest to replace and is a good place for the most variable component, such as cDNA, as seen in our plasmid system. Carefully designed LAPs and RAPs can serve as templates for more complicated LAPs and RAPs.

Progress in molecular biological techniques has simplified a variety of procedures and has accelerated research progress in many fields. Constructing multicomponent plasmids remains a time-consuming step that requires experience and skill. Our wrapper arms strategy simplifies the procedure and provides a framework for the construction of complicated designer plasmids for a variety of applications.

REFERENCES


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