Abstract

Introduction
To overcome the shortcomings of traditional cloning methods, ligase-free methods or ligation independent methods were developed to provide more choices for technicians. In this article, the history, mechanism and application of four in vitro ligase-free methods are reviewed in detail: (i) ligation independent cloning, (ii) gateway recombinational cloning, (iii) uracil-DNA glycosylase and (iv) restriction free cloning.

Conclusion
These ligation independent cloning tools are simple, fast and efficient tools for high-throughput cloning or long expression vector assembly.

Introduction
Since the plasmid was found and modified as a useful DNA vector, gene cloning has become a basic and indispensable tool in the field of biological research, especially in molecular biology. Saiki et al. invented the polymerase chain reaction (PCR) in 1988, which brought about a revolution in genome research. Then on, so many cloning methods based on PCR were carried out. Gene engineering techniques have burst onto the scene in the last three decades; meanwhile, hundreds of groups have utilised or improved these techniques in the fields of biological research. Generally speaking, the basic work involved in gene engineering is operating DNA molecules in vitro by assembling target DNA fragments into suitable vectors, mostly plasmid vectors. For example, somatic gene targeting, which includes somatic gene knock-out and gene knock-in, involved in the construction of the adeno-associated virus (AAV) vector, recommends left and right homologous arms insertion. Both homologous arms are often amplified from the genome by PCR and link with the selectable resistance gene flanked by LoxP sites, step by step. However, the quest to find a timesaving and high-throughput technique is tough work as general plasmid vectors are not designed for use in simultaneous insertion. The most common technique or the so-called traditional method is ‘restriction-ligation’. Though various restriction enzymes have been found and artificially modified by a lot of laboratories, the application of this method is still restricted. The limitations of this method are obvious; it calls for the well-defined sequence information of the vector and target DNA, and sometimes it becomes difficult to find suitable restriction enzymes in the target DNA. Moreover, false positive clones are common because of the universal nucleotide ligation, especially null vector self-ligation by the ligase. Hence, cloning of genes or other DNA segments on a large scale seems to be laborious. Nevertheless, some other tools have been created to reduce the complexity and laboriousness of the task.

When someone attempts to screen an unknown interaction between the proteins of the expression files and a protein of interest, yeast dual hybridisation is often used. However, building a good library which involves thousands of cDNAs is a laborious process. Additionally, several novel gene knock-out methods are popular these days, such as transcription activator–like effector nucleases (TALEN) and zinc-finger nucleases (ZFN). The assembly of TALEN and ZFN vectors involves long modular cDNA rearrangement. In our practice, construction of these vectors by traditional methods is time-consuming and inefficient. Many efforts have been made to achieve better means to overcome the shortcomings of the ‘restriction-ligation’ method. Ligase-free strategies, including ligation independent cloning (LIC), gateway recombinational cloning, In-Fusion kit, uracil-DNA glycosylase (UDG), sequence and ligation independent cloning (SLIC), restriction free (RF) cloning, etc., were explored; successful results obtained from these methods are uncountable. These methods serve as simple, quick and efficient choices for high-throughput cloning or long expression vector assembly.

In this critical review, we take a look at the history, mechanisms and applications of four ligase-free cloning methods: (i) LIC, (ii) gateway recombinational cloning, (iii) UDG and (iv) RF cloning.

Ligation independent cloning
In a narrow sense, LIC is an exclusive name, referring to a method that uses T4 DNA polymerase or exonuclease III (EXOIII). In the broad sense, LIC refers to a cloning method that does not involve the traditional ligation procedure.

The LIC method was first established and defined as an exclusive method by Aslanidis and Jong. This method is based on pairs of unique primers and the (3’-5’) exonuclease activity of T4 DNA polymerase. Firstly, primers were used to modify the
vector with the 5’ end lacking dGMP or a pair of complementary primers was used. The PCR products were amplified using a pair of additional, unique, 12-base primers. These 12 additional bases were added onto the 5’ end of the primers lacking dCMP. After being treated by the (3’-5’) exonuclease activity of the T4 DNA polymerase in the presence of dCTP, the PCR products generated 12-base overhangs, which were complementary to the vector treated by the T4 DNA polymerase in the presence of dGTP. The T4 DNA polymerase shows a (3’-5’) exonuclease activity and a (5’-3’) polymerase activity, so in the presence of dCTP or dGTP, the 13th base is lost without deeper excision. Since the single strand tails are easy to pair with their complementary tails, circularisation occurs easily when mixing the treated vector and the PCR product together under the right conditions. This technique for cloning is much faster, simpler and more efficient than the ‘restriction-ligation’ method. Many other researchers use this method to generate vectors with genes of their interest11-14.

Some improvements have also been made to facilitate its application. Tachibana et al.15 used a two-step T4 DNA polymerase-treated method to generate sticky ends, and the PCR products were re-annealed, not digested by T4 DNA polymerase to create single strand tails. A high-throughput method known as the SLIC, which is a modification of the LIC method, was reported in 20079. After adding RecA, a well-known recombinase, an efficient process of recombination of the PCR products was achieved. Recently, Schmid-Burgk et al.16 reported a TALEN assembly method using LIC, based on EXOIII, by introducing two PCR fragments simultaneously into a modified AAV vector that made construction of somatic gene knockout vectors a time-saving and efficient procedure2. It might be noted that the (3’-5’) exonuclease activity of EXOIII performs well only when catalysing the substrates with 5’ end tails or blunt ends, so the PCR products must be amplified using the Pyrococcus furiosus DNA polymerase or other polymerases without transferase activity at the 3’ end.

The mechanism of T4 DNA polymerase and exonuclease III in LIC was carefully studied2; it showed evidence that LIC is a flexible strategy. It is also recommended that no rigid sequence compatibility, gaps or mismatches can be repaired in the host bacteria. Some other improvements were made to generate PCR products with overhangs in the absence of exonuclease treatment. One method employed the usage of 1,3-propanediol in defined positions, so the Taq polymerase stopped chain reactions when encountering with non-base residues18. Another method introduced a protected nucleotide with a photolabile protective group into the PCR primers, leading to a new procedure named as caged PCR20.

**Gateway recombinational cloning**

Gateway recombinational cloning was first carried out by Walhout et al.5. The gateway technique is a universal recombinational cloning method based on the site-specific recombination system of the λ bacteriophage. Gateway is comprised of the BP reaction and LR reaction, both in vitro recombinations. For example, to clone a gene of interest into an expression vector, an entry clone must be constructed first. PCR primers with additional attB sequences were used to amplify the target DNA. By the recombinational reaction attB × attP → attL × attR, which was mediated by BP clonase, the gene of interest flanked with attB could be inserted into the donor vector, replacing the CcdB gene, becoming a new vector called as the entry clone. The lethal gene CcdB in the donor vector kills its host bacteria (such as Top10 or DH5α), so the unrecombinational vector cannot be duplicated. This method is commercially available in Invitrogen (Carlsbad, CA, USA)34. Gateway has shown good application in the construction of high-throughput vectors in many laboratories20-22, and it is a widely used tool to generate cDNA libraries. cDNAs can be easily cloned between different types of vectors, without sequencing certification.

However, assembly of long modular cDNAs is still limited because of the rare joint choices. Suzuki et al.23 reported an ‘N- and C-term’ gateway cloning method by which two PCR products could be inserted simultaneously, relying on the homologous ends for bacteria recombination. Invitrogen (Carlsbad, CA, USA) further developed a new strategy called the ‘Gateway MultiSite recombinational cloning’ method, intending to solve the aforementioned limitations of the gateway cloning method24. This kit uses an upgraded mixture of recombinant enzymes known as clonase II. By utilising five different att sites, up to four DNA fragments could be cloned into a destination vector in one reaction. Some groups have created complete protocols for the insertion of several DNA segments using this method of
cloning. For instance, Sone et al. present the Gateway MultiSite cloning of pDEST vectors for use in *Saccharomyces cerevisiae*. Nevertheless, if we do want reliable multiple cDNA assembly, the Gateway strategy still needs to be improved, maybe by employing more att sites or by aligning with other gene engineering methods.

**Uracil-DNA glycosylase cloning**

By introducing dUMP instead of dTMP into the primers, scientists have developed novel techniques for vector construction. Early in 1991, Nisson et al. found that UDG was a good tool to generate cohesive ends. They synthesised primers with four dUMPs on the 3’ end for amplifying the vector and target DNA. UDG cleaves the N-glycosylic bonds between other deoxyriboses and uracil in double-stranded DNA (dsDNA), so the adjacent oligonucleotides are unstable and float away from the dsDNA, which leads to the formation of long tails on both the 5’ ends of the PCR products. However, this procedure is not very efficient and economical as the protruding single-stranded flaps are still attached onto the recombinant products, and the synthesised primers with dUMPs are much more expensive than common primers. Some genius trials were later conducted to improve the efficiency in the generation of sticky ends. For effective breaking-off of flaps, another enzyme was previously added to the reaction system. This enzyme mixture (together with UDG) was named as uracil-specific excision reagent (USER™), which is commercially produced by New England Biolabs (Ipswich, Massachusetts, USA). The enzyme is a DNA glycosylase-lyase endonuclease VIII. UDG excises a uracil base, forming an abasic site and promotes strand separation. The lyase activity of endonuclease VIII breaks the phosphodiester backbone at the 3’ and 5’ ends of the abasic site so that base-free deoxyribose is released. Geo-Flores et al. used USER™ to generate long fusion open reading frames (ORFs); although SpeI and EcoRI were employed to create long sticky ends on the vector and PCR products, they were not as long as we wished them to be. During the same time, by inserting a designed cassette into the vector, Bitinaite et al. reported a good way to generate long tails on vectors using the Nt.BbvCl and XbaI exonucleases. Another group also reported a similar idea. This skill enabled the universal application of the USER™ system, even in high-throughput applications such as gene library construction.

**Restriction free cloning**

RF cloning is a simple PCR method for cloning; it is a method developed from circular PCR. Van den and Lowe used the PCR procedure alone, constructing many expression vectors; the results from western blotting demonstrated that these constructions were impressively successful. The procedure was employed as follows. Firstly, primers flanked by unique restriction enzymes were used to amplify the genes of interest. A vector isolated from a Dam™ strain of bacteria (the most engineered bacterial strain, the DH5α) was previously modified and used as follows. Firstly, primers flanked by unique restriction enzyme Nt.BbvCI 32. The readers can refer to the cited references for more information.

In summary, LIC was intended to solve two puzzles caused by the traditional cloning method. The first one is high-throughput cloning. The second one is long modular cDNA joining. Utilising restriction enzymes with rare recognition sites enable high-throughput cloning or long modular cDNA generation when aligned with other tools such as the Golden Gate. The LIC and UDG methods provide appropriate means for the generation of long cohesive ends, method was the *Pfu Turbo* because this enzyme performs with high fidelity and does not add dATP onto the PCR products. Agroup from Israel performed simultaneous cloning of several DNA fragments using the RF cloning technique and facilitated recombinant protein expression in the *Escherichia coli* system.

An online tool is also available for the design of RF cloning projects.
subsequently leading to the automatic pairing of long complementary sticky ends; the repair mechanism of the host bacteria fills up those gaps after transformation. By using these methods, DNA with unclear sequence information can be easily cloned; it can also enable zero-gap conjugation. All the three methods, LIC, UDG and RF-cloning, employ PCR to amplify DNA segments. Mutation cases are often caused by PCR, and this is a concern that must be duly addressed. Although high fidelity polymerases that are commercially available are purchased, mutations in PCR products still cannot be eliminated. Moreover, in some cases, ORFs tolerate no mutations. Sequencing is a good way to guarantee the precision of the PCR-generated plasmids. For TALEN or ZFN assembled vectors, which consist of 15(TALEN) and 5(ZFN) or more recognition DNA sequence repeats, sequencing is an inefficient tool to certify the wanted clones. Hence, they still pose limitations in the prevention of potential mutations in the target DNA.

The Gateway system is a significant tool for library cloning, by clonase-mediated recombination. DNA mutation is not generated during the Gateway method. However, for the assembly of long, modular, protein-expression vectors, further research must be conducted.

Conclusion
LIC is a powerful in vitro cloning tool for high-throughput cloning or long expression vector assembly. As outlined above, some limitations of LIC methods still remain to be addressed.

Abbreviations list
AAV, adeno-associated virus; dsDNA, double stranded DNA; EOXIII, exonuclease III; LIC, ligation-independent cloning; ORF, open reading frame; PCR, polymerase chain reaction; RF, restriction free; SLIC, sequence and ligation independent cloning; TALEN, transcription activator–like effector nucleases; UDG, uracil-DNA glycosylase; USER, uracil-specific excision reagent; ZFN, zinc-finger nucleases.

References
22. Atanassov II, Atanassov II, Etchells JP, Turner SR. A simple, flexible and efficient PCR-fusion/Gateway cloning procedure for gene fusion, site-directed mutagenesis, short sequence insertion