Realizing directional cloning using sticky ends produced by 3′-5′ exonuclease of Klenow fragment

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The Klenow fragment (KF) has been used to make the blunt end as a tool enzyme. Its 5′-3′ polymerase activity can extend the 5′ overhanging sticky end to the blunt end, and 3′-5′ exonuclease activity can cleave the 3′ overhanging sticky end to the blunt end. The blunt end is useful for cloning. Here, we for the first time determined that a sticky end can be made by using the 3′-5′ exonuclease activity of KF. We found that KF can cleave the blunt end into certain sticky ends under controlled conditions. We optimized enzyme cleavage conditions, and characterized the cleaved sticky ends to be mainly 2 nt 5′ overhang. By using these sticky ends, we realized ligation reaction *in vitro*, and accomplished cloning short oligonucleotides directionally with high cloning efficiency. In some cases, this method can provide sticky end fragments in large scale for subsequent convenient cloning at low cost.

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1. Introduction

DNA polymerases replicate genomes for cell growth and cell division. Despite their important role *in vivo*, they have been also used as tool enzymes, such as *pfu* polymerase for PCR, Klenow fragment (KF) for nucleic acids labelling, *phi*29 polymerase for rolling circle amplification (Yang *et al.* 2005; Berrow *et al.* 2009). KF is the large fragment of polI polymerase of *E coli*. It is among the first discovered DNA polymerases and is now commonly used as a tool enzyme. KF bears two kinds of enzyme activities according to different domains: DNA polymerase activity and 3′-5′ exonuclease activity (Freemont *et al.* 1988; Beese and Steitz 1991). The 3′-5′ exonuclease activity is important for the fidelity of DNA polymerase (Eger *et al.* 1991; Carver *et al.* 1994; Zhang *et al.* 2005). By cutting mismatched base pairs during polymerization reaction, mutation rate is greatly reduced. Although some of characteristics of 3′-5′ exonuclease activity have been explored, such as the effect of temperature, dNTP, and substrates(Brutlag and Kornberg 1972), little effort has been focused on the digested products. Both the polymerase activity and the exonuclease activity are applied mainly for blunt end making, and the blunt end can be used in following cloning steps. Studies have also suggested that deficient in 3′-5′exonuclease, a 3′ adenine nucleotide overhang sticky end can be made (Clark *et al.* 1987). This kind of sticky end is formed by terminal deoxynucleotidyl transferase activity, and is not reported of the application in cloning. Besides the 3′A

Keywords. Directional cloning; exonuclease; Klenow fragment; sticky end

Abbreviations used: DTT, dithiothreitol; IPTG, Isopropyl β-D-1-thiogalactopyranoside; KF, Klenow fragment; KPB, potassium phosphate buffer; ODN, oligodeoxyribonucleotide; PAGE, polyacrylamide gel electrophoresis; TdT, terminal deoxynucleotidyl transferase

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sticky end, little has been explored to use the 3′-5′ exonuclease cleavage of KF to make sticky end, let alone to use the sticky end made by KF exonuclease in cloning methods.

Traditional sticky end cloning methods are dominated by restriction endonucleases (RE). RE can recognize certain DNA sequence and cut at the cleavage site. Two kinds of cleavage ends can be made: blunt end and sticky end. The latter is most commonly used in cloning, because it has higher ligation efficiency and can make directional cloning. However, RE-dependent sticky-ended cloning needs certain RE recognition sequence, and in some instances, the recognition sequence should be included into cloning substrates, such as PCR primers. Although many REs are commercially available which makes cloning convenient, the costs are still high for many REs. Besides RE-based cloning, terminal deoxynucleotidyl transferase (TdT) activity of some polymerase leads to another commonly used sticky-ended cloning method: TA cloning (Clark 1988; Hu 1993; Motea and Berdis 2010). Although it is convenient for PCR product cloning, TA cloning needs to prepare special vectors and is not a directional cloning method (Zhao Yaofeng 2009; Zhou and Gomez-Sanchez 2000).

Here, by using KF we tested the possibility if a certain desired sticky end product can be produced by the digestion of KF. We found in certain reaction conditions, the 3′-5′ exonuclease activity of KF can make 2 nt sticky ends. Furthermore, this leads to a new kind of cloning method, and by using these sticky ends, we successfully cloned oligonucleotides into pET28a plasmids. This strategy is cost-effective, and has high positive rate in directional cloning.

2. Materials and methods

2.1 *Materials and reagents*

Plasmid pET28a, bacterial strains DH5α and BL21 were kept in our lab. Oligonucleotides were synthesized from Genscript Corporation (Nanjing, China). The stock solutions of oligonucleotides were prepared in concentration of 100 μM and were stored at −20°C.

Pfu DNA polymerase, DNA Gel Extraction Kit and Plasmid Miniprep Kit were purchased from Tiangen Biotech Corporation (Beijing, China). Restriction endonucleases (*Eco*RI, *Hind*III and *Hpa*I), DNA ligation kit, and *Taq* DNA polymerase were obtained from TaKaRa Biotechnology Company (Dalian, China). Ni-NTA column was from GE Healthcare.

2.2 *Cloning, expression and purification of KF*

KF gene fragment were constructed by PCR from DH5α. Upstream primer is 5′- CGGAATTCGTGATTTCTTATGACAACTACGTCAC-3′ carrying *Eco*RI cleavage site. The downstream primer is 5′-GAAAGCTTGTGCGCCTGATCCCAGTTTT-3′ carrying *Hind*III cleavage site (the cleavage sites are underlined). PCR product was cleaved and purified to be cloned into pET28a plasmid.

Recombinant plasmids of pET28a-KF were transformed into BL21 competent cells. Cells cultured in LB-containing kanamycin were induced by 5 mM IPTG when OD600 of cell culture solution reached 0.6. After continuous incubation for 4 h at 37°C, cells were collected by centrifugation. BL21 cells were resuspended in lysis buffer and were lysed by ultrasonication. Cell lysates were centrifuged. The supernatant was purified by Ni-NTA column according to the GE product manual. The eluent was dialysed, and the protein solution was prepared in buffer containing 50 mM KPB pH 6.5, 10 mM β-Mercaptoethanol, 50% Glycerol, and was stored at −20°C.

2.3 *Exonuclease reaction and gel shift assay*

Substrate oligonucleotides (ON) 0.5 μM were prepared in 10 μL reaction buffer containing 10 mM Tris pH 7.5, $MgCl_2$ 7 mM, and DTT 0.1 mM. Cleavage reaction was started by adding 0.3 μM DNA polymerase at 24°C for 10 min. The reaction was stopped by adding loading-buffer-containing 5 mM EDTA, 6% Glycerol, 0.006% Xylene Cyanol FF, and 0.01% Bromophenol Blue. The products were analysed by 18% nondenaturing PAGE and fast silver stain method as described (Zhao and Guan 2010).

2.4 *ON ligation using KF cleaved sticky ends*

Cleavage reaction by Klenow fragment is mentioned in Section 2.3. The cleaved product (2.5 pmol) and synthesized ligation substrates (2.5 pmol) were ligated by 175 Units of T4 DNA ligase in 10 μL reaction buffer containing 45 mM Tris pH 7.6, $MgCl_2$ 7 mM, DTT 5 mM, and ATP 2.5 mM. After 10 min at 24°C, ligation reaction was stopped by adding loading-buffer-containing 5 mM EDTA, 6% Glycerol, 0.006% Xylene Cyanol FF, and 0.01% Bromophenol Blue. The ligation products were analyzed by 18% nondenaturing PAGE and fast silver stain method as described (Zhao and Guan 2010).

2.5 *Cloning ON in KF cleaved vectors*

For non-directional cloning, plasmid pET28a was cut by *Hpa*I, and was purified. Linearized plasmid 0.4 ng/μL, and was cleaved by 0.06 μM KF, and was ligated with ON. Ligation product was transformed into DH5α. For directional cloning, plasmid pET28a was cut by *EcoR*I and *Hpa*I. Linearized plasmid was cleaved by KF, and was

ligated with ON. Ligation product was transformed into DH5α. Positive clones verified by PCR. For forward insertion, we used primer-F and insert-a. For reverse insertion, we used primer-F and insert-s. At least three positive clones were verified by sequencing for both forward and reverse insertions.

3. Results

3.1 *Cloning, expression and purification of KF*

We designed two specific primers to amplify KF. Upstream and downstream primers carry *Eco*RI and *Hind*III recognition sequence respectively. The PCR product was purified, and the purified PCR product was directionally cloned into pET28a through two RE cleavage sites. The recombinant plasmids of positive clones were identified by *Eco*RI and *Hind*III cleavage. Figure 1A shows cleaved products have two bands: one is vector the other is insertion KF DNA which is expected to be 1.8 kb.

By IPTG induction, the expression of KF in *E. coli* was obvious (figure 1B). For the recombinant protein tagged with hexa-histidine, a Ni-NTA column was used for purification. Most bacterial proteins were removed, and the KF protein was purified as a main band in SDS-PAGE (figure 1C). The recombinant KF protein is estimated to have a molecular weight of 68 kDa and was confirmed by SDS-PAGE.

3.2 *3′-5′ Exonuclease activity of Klenow fragment*

We tested the 3′-5′ exonuclease activity of purified KF by using gel shift assay. Two kinds of substrates were used. For single-stranded substrate ODN7, the cleavage was unaffected by dNTP (figure 2A). However, for duplex substrate D20, the cleavage was affected not only by dNTP but also by temperature. At room temperature for 30 min, one can not see obvious digestion, and at 37°C, cleavage only occurred in the absence of dNTP (figure 2B). These conditions directed us to the following experiments.

3.3 *Sticky end produced by 3′-5′ exonuclease activity of Klenow fragment*

We wondered whether a sticky end can be made by partially digestion of 3′-5′ exonuclease of KF. For sticky-end identification, we used short oligonucleotide substrate and native PAGE as described previously (Zhao and Guan 2010; Zhao 2013). The optimal digestion condition for sticky end production was determined by gel shift assay (figure 3). We used D20-2 as a sticky-end duplex control. Blunt-end substrate D20 was digested into the sticky end of D20-2 in 5 min. After 10 min reaction, the bands of sticky-end products became weak and disappeared. Consequently, we can acquire D20-2 sticky-end products by KF in a reaction time ranging from 5 to 10 min.

3.4 *Characterization of sticky ends made by KF cleavage (ligation reaction in vitro)*

Although the band of digested product of D20 showed the same band position of D20-2, the exact length of sticky end needs further verification. To testify the sticky end produced by KF, we carried out ligation reaction. The partially digested oligonucleotides were reacted with series of sticky-ended substrates with 5′ overhangs ranging from 1 to 4 nt (figure 4A). The ligation products were determined by gel shift assay. Results showed that the mostly produced sticky end is 1-2 nt long sticky end (figure 4B). This *in vitro* ligation experiment showed the feasibility for ligation of sticky end produced by KF cleavage.

3.5 *Non-directional cloning using sticky ends made by KF*

To testify producing sticky end in plasmid vector by KF, and the ability to cloning oligonucleotides, we designed the

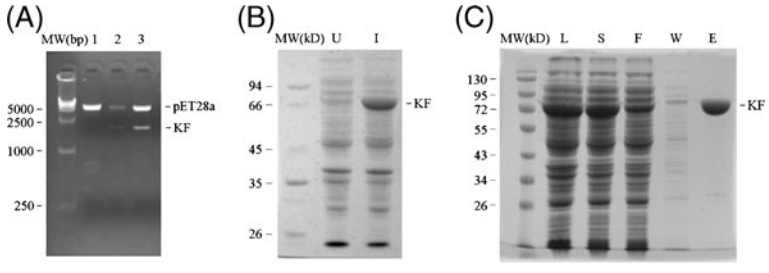

Figure 1. Cloning, expression and purification of KF. (**A**) Recombinant plasmid identification of three clones by double restriction endonucleases digestion. (**B**) KF expression induced by IPTG. (**C**) KF purification by Ni-NTA column.

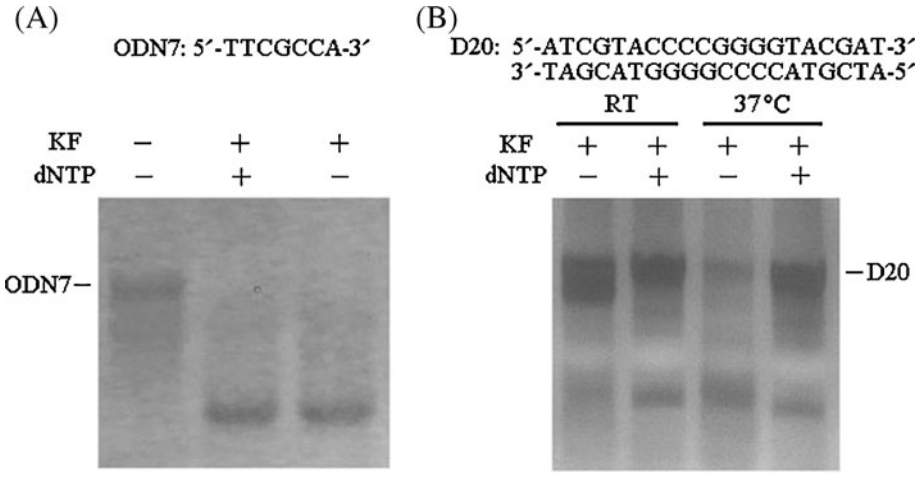

Figure 2. Activity of 3′-5′ exonuclease affected by substrate, dNTP and temperature. (**A**) Single-stranded substrate. (**B**) Duplex substrate.

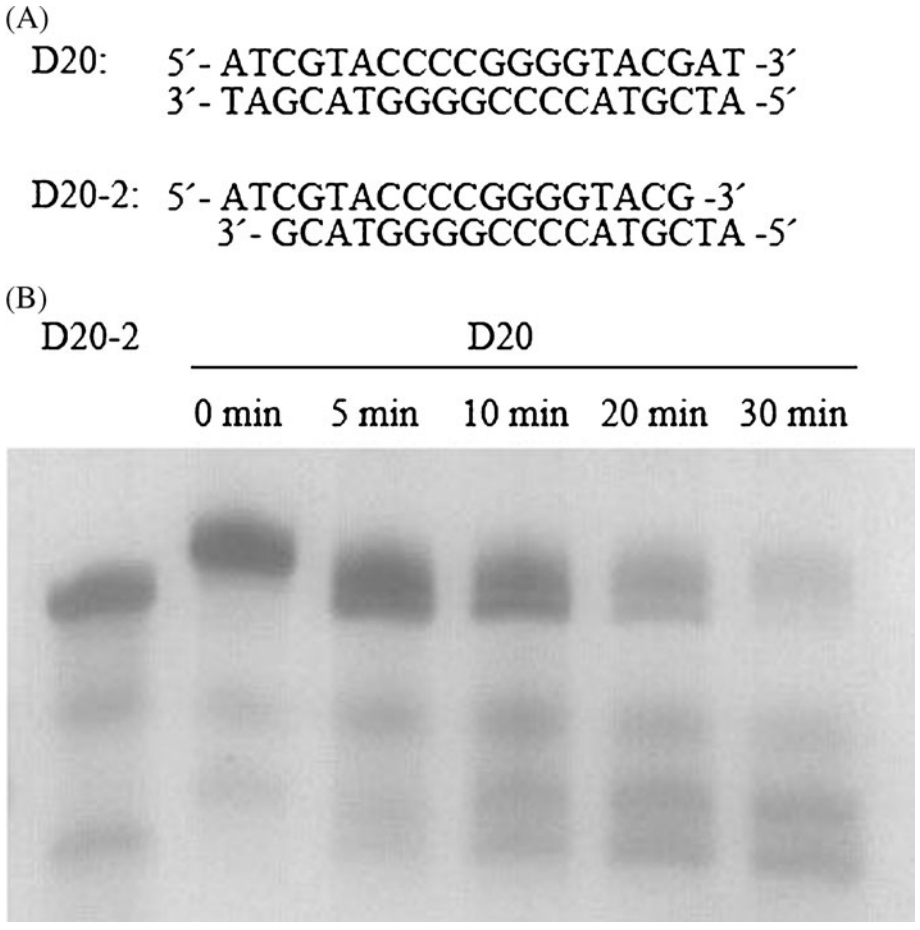

Figure 3. Cleavage of D20 by 3′-5′ exonuclease activity of KF. (**A**) D20 duplex as substrates and D20-2 duplex as a control of digested D20 by KF. (**B**) Native PAGE of D20 digestion product by KF.

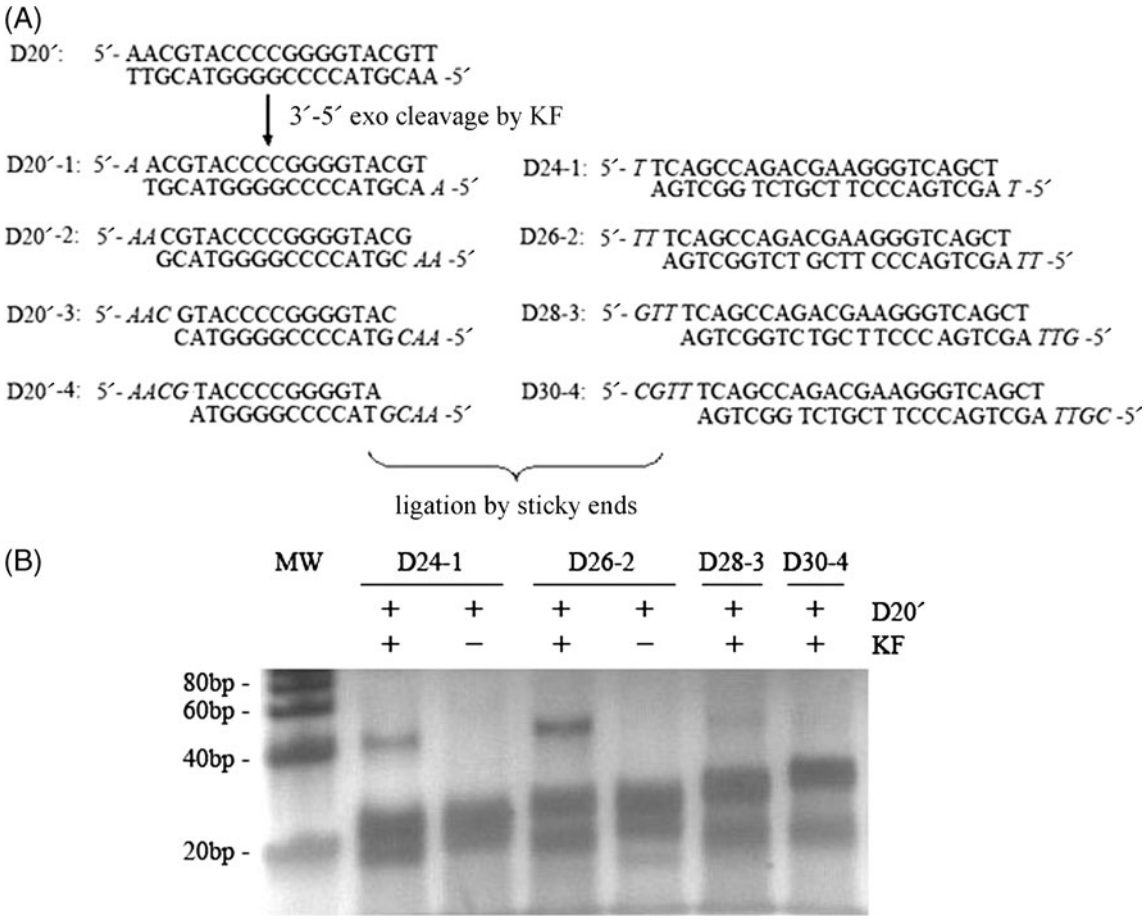

Figure 4. Sticky end ligation reaction using digested products of D20′ by KF.

following experiment. Plasmid pET28a was linearized by *Hpa*I to form blunt ends. Then KF was added to carry out partially 3′-5′ exonuclease cleavage reaction to make sticky ends. The insertion fragment bore sticky end complementary with the sticky end in the vector produced by KF. The digested vector (pET28a′) and insertion fragment (D26-2) were ligated and transformed into DH5α competent cells (figure 5, left column).

We employed PCR to select positive recombinant clones. We used two pairs of primers to discriminate forward cloning and reversed cloning. The upstream primer was a universal primer about 300 bp from insertion site in vector plasmid. The insertion substrate was formed by a sense oligonucleotide and an antisense oligonucleotide. Consequently, the antisense ON was used as downstream primer for forward insertion detection (insert orientation: A→B) and the sense ON was used as downstream primer for reverse insertion detection (insert orientation: B→A) (figure 6). The positive clones selected by PCR were verified further by DNA sequencing. Results showed that we obtained 10 forward insertion recombinants and 9 reversed recombinants from 38 clones (figure 7; table 1). DNA sequencing showed the right insertion sequences and sticky-end ligation sites (supplementary figure 1A, B).

3.6 *Directional cloning using sticky ends made by KF*

To verify whether we can realize directional cloning using KF cleavage, we carried out the following experiments. Plasmid pET28a was linearized by *Eco*RV and *Hpa*I to form blunt ends. Then KF was added to carry out partially 3′-5′ exonuclease cleavage reaction to make two different sticky ends. The insertion fragment bore two different sticky ends respectively complementary with the two sticky ends in vector produced by KF. The digested vector (pET28a′′) and insertion fragment (D26-2′) were ligated and transformed into DH5α competent cells (figure 5, right column). The recombinant clones

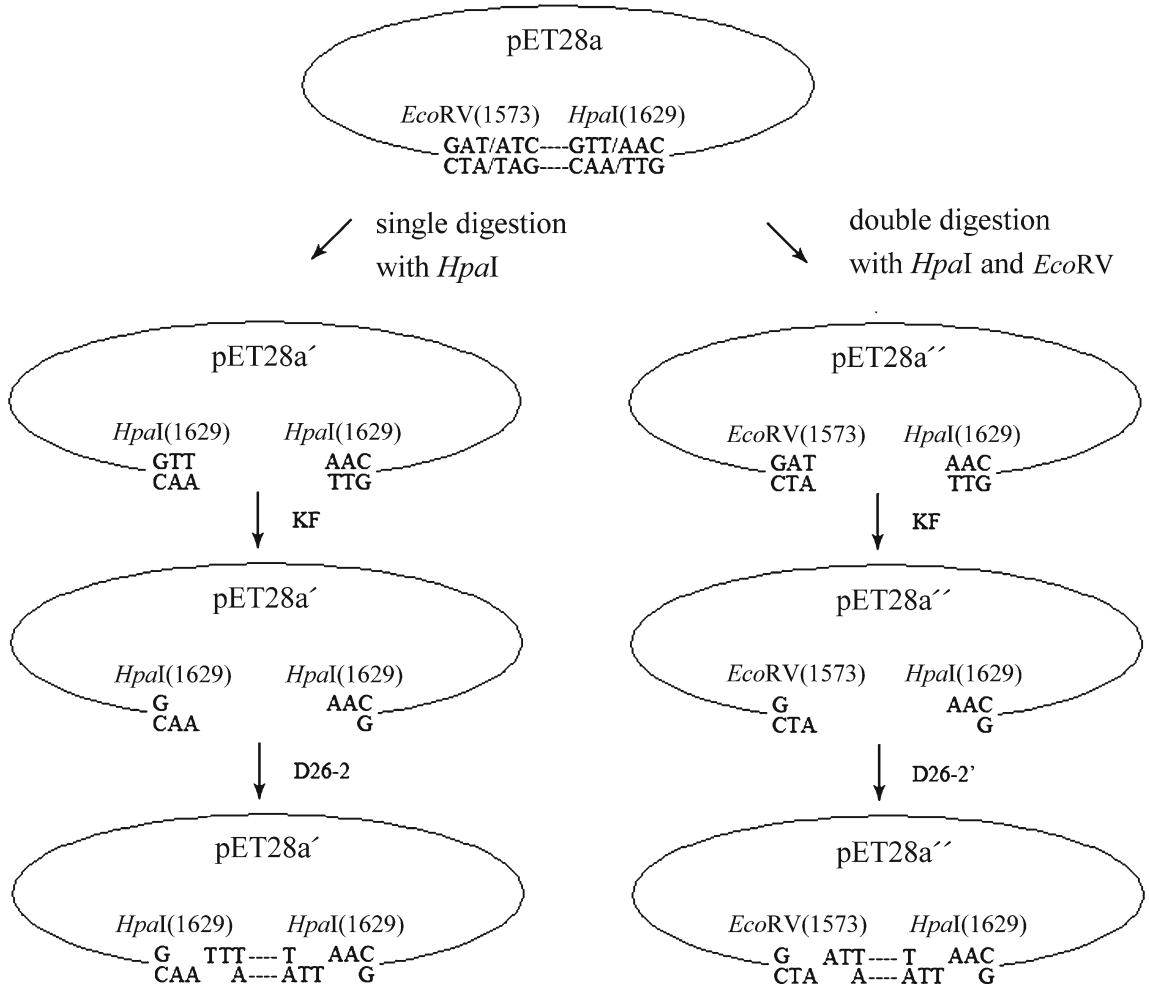

Figure 5. Schematic of cloning experiments using KF-made sticky ends. pET28a′: *Hpa*I single digested vector; pET28a′′: *Hpa*I and *EcoR*V double digested vector; left column: non-directional cloning; right column: directional cloning.

were selected by PCR and identified by DNA sequencing. Results showed that we obtained 19 forward insertion recombinants and 2 reversed recombinants from 51 clones (figure 8; table 1). DNA sequencing showed the right insertion sequences and sticky-end ligation sites (supplementary figure 1C).

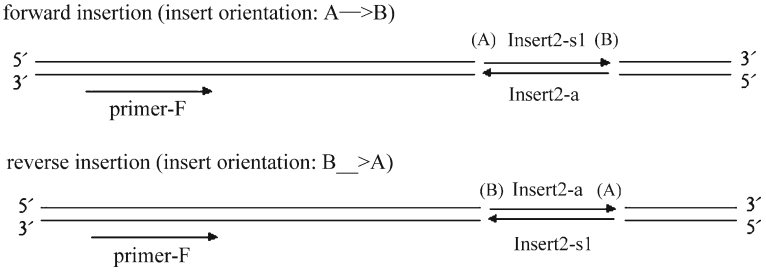

Figure 6. Schematic of identifying forward or reverse insertions by PCR. Insert is composed of oligonucleotides insert2-s1 and insert2-a. The ends of insert duplex are labeled with 'A' and 'B'. The orientations of insertion are demonstrated as 'A->B' for forward insertion and 'B->A' for reverse insertion.

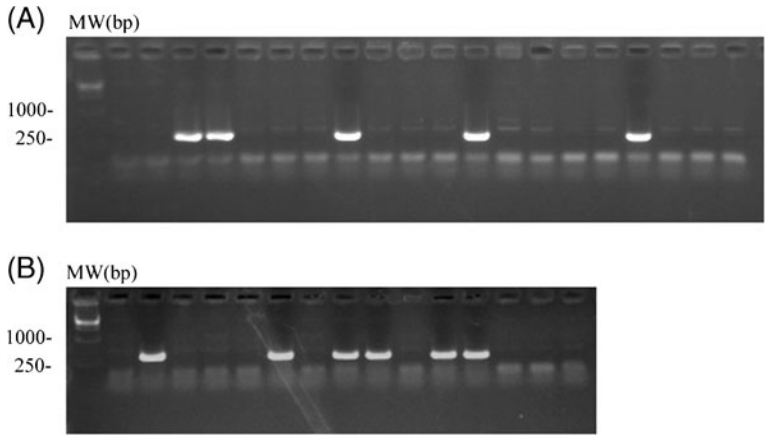

Figure 7. Forward (**A**) or reverse (**B**) insertion identified by PCR.

4. Discussion

4.1 *Sticky end produced by KF*

KF bears two types of enzyme activity: 5′-3′ polymerase activity and 3′-5′ exonuclease activity. The 5′-3′ polymerase activity can fill the 5′ overhang sticky end by primer extension reaction. The 3′-5′ exonuclease activity can digest the 3′ overhang sticky end to the blunt end. Therefore, KF is a tool enzyme to make the blunt end. With the cooperation of 3′-5′ exonuclease activity, polymerase domain synthesizes DNA with high fidelity. The 3′-5′ exonuclease activity of KF is influenced by several factors. High dNTP concentration inhibits digestion of KF. Without dNTP, KF bears no polymerization activity but only 3′-5′ exonuclease activity, and can digest duplex nucleic acids in 30 min (figure 3). However, the sticky ends of digested products by exonuclease of KF were seldom explored.

The length of nucleic acid is easy to be determined, such as by denatured electrophoresis. However, to determine whether the end of a duplex is a sticky end or blunt end is not easy. In a previous study, we described a simple effective method to discriminate the sticky end from the blunt end by using native PAGE (Zhao *et al.* 2013). Here, we also characterized duplex ends by using this method. We found that the cleaved products with certain length of sticky ends can be obtained under controlled conditions (figure 3). We digested D20 to a majority of 2 nt overhang products which had the same band position as D20-2 in 37°C for 5 min. When elongating reaction time over 10 min, 2 nt overhang products disappeared quickly. Considering a 2 nt overhang sticky end was suitable for sticky-end ligation, we prepared four duplex oligonucleotides bearing sticky ends. The sticky ends ranged from 1 to 4 nt overhang, which was expected to be complementary and to be ligated with digested D20′ (figure 4A). Gel shift assay of ligation products showed only duplex D24-1 and D26-2 can be ligated with digested D20′, suggesting D20′ must be digested into D20′-1 and D20′-2 which bears 1 nt and 2 nt overhangs respectively, and the majority product was D20′-2 (figure 4B). This was in accord with the result of gel shift assay in figure 3.

After verifying the sticky ends from KF cleavage, we designed a cloning strategy to clone oligonucleotides in plasmids. In this strategy, plasmid pET28a was used as a model. First, we tried non-directional cloning. After pET28a was linearized by restriction endonuclease *Hpa*I, the blunt ends of linearized plasmid were cleaved by KF to make 2 nt overhang sticky ends (5′AA…). Then D26-2 (bearing sticky ends of 5′TT…) was added and ligated with plasmid by T4 DNA ligase. Different from non-directional cloning, in directional cloning, the plasmid was linearized by two restriction endonucleases: *Eco*RV and *Hpa*I. After cleaved by KF, pET28a bears two different 2 nt sticky ends (5′AT… and 5′AA…). Then D26′-2 (bearing sticky ends of 5′TT…

Table 1. Positive clones identified by PCR

	Total cloning number	Forward insertions	Reverse insertions	Ratio of forward vs reverse
Non-directional cloning	38	10	9	1:1
Directional cloning	51	19	2	10:1

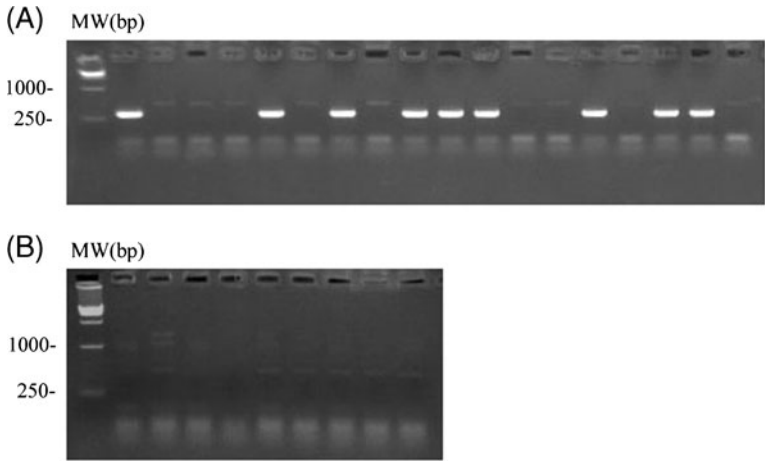

Figure 8. Forward (**A**) or reverse (**B**) insertion identified by PCR.

and 5′TA…) was added and ligated with plasmid by ligase (figure 5).

We used PCR to identify positive insertion clones. Both forward insertion and reverse insertion shared the same upstream primer. For identifying forward insertion (insert orientation: A→B), primer-F and insert-a were used. For identifying reverse insertion (insert orientation: B→A), primer-F and insert-s were used (figure 6). This design succeeded in identifying both forward and reverse insertion clones, which were further verified by DNA sequencing.

In non-directional cloning, we picked 38 clones totally. PCR identified 10 forward insertions and 9 reverse insertions. The ratio of forward and reverse insertions was 1:1. In directional cloning, we picked 51 clones totally. PCR identified 19 forward insertions and 2 reverse insertions. The ratio of forward and reverse insertions was 10:1 (table 1). This showed this cloning method has high efficiency and realized cloning in direction. In the case of directional cloning, 2 reverse clones of total 21 positive clones were identified unexpectedly. Sequencing of these two clones showed correct ligation. This may be temporarily ligated in wrong sequence, but can be repaired in cells later.

4.2 *Exonuclease-dependent sticky-end cloning method*

Methods for making sticky ends typically include RE cloning and TA cloning. They are still mostly used cloning methods. Different REs can recognize different DNA sequences and cleave DNA to form various sticky ends. To directionally clone a DNA fragment into a vector, it needs at least two different REs and their recognition sequences in both vector and insertion DNA. With more fragments insertion, even more REs are needed (An *et al.* 2010). Many REs are available for the convenient cloning, but many of them are not cost-effective. Moreover, internal cleavage site of RE should be avoided during RE cloning. Furthermore, if RE recognition site locates at the ends of duplex, such as PCR primers, several additional protection nucleotides should be added. Considering different REs need their optimal buffers, several REs in one reaction must face a problem of buffer incompatibility.

TA cloning does not need REs, recognition sequences and terminal protection nucleotides of PCR primers; therefore, it is convenient for PCR product cloning. Although the cloning vectors need to be prepared as 1 nt T 3′ overhang by special treatment, the once-for-all preparation is convenient for every subsequent PCR cloning. However, the shortcoming is non-directional. Moreover, T/A is the only sticky-end type; hence, multi-fragments ligation in one reaction is almost impossible.

Several exonucleases have been used for cloning (Kaluz *et al.* 1992; Yang *et al.* 1993). The multiple-cloning by the 3′-5′ exonuclease of T4 DNA polymerase showed the advantage of exonucleases in cloning (YunHua *et al.* 2006). The 3′-5′ exonuclease activity of T4 DNA polymerase was used to make sticky ends; however, the sticky ends were not homogeneous, and the vector joined insertions through long overhangs hybridizing but not through ligation by ligase (Yang *et al.* 1993). Another T4 polymerase-based method has used certain dNTP to make sticky ends, but it needs palindrome of G/Cs or A/Ts (Stoker 1990). ExonucleaseIII has also been used to make sticky ends for cloning; however, the exact length of sticky end is not determined (Kaluz *et al.* 1992). Nucleotide analog has also been introduced into this kind of strategy. PCR products digested by lambda exonuclease and stopped by phosphorothioate can precisely make overhangs (Liu and Liu 2010). Phosphorothioate linkage can also be protected from 5′-3′ exonuclease cleavage of *pol*I (Howland *et al.*

2011). ALthough precision sticky end can be made in this strategy, nucleotide derivatives should be introduced into cleavage substrates. Besides the nucleases mentioned above, the capability of KF exonuclease in cloning has not been examined to date. In this study, we used 3′-5′ exonuclease of KF as a new exonuclease for sticky-end cloning, and expanded the application of this tool enzyme from traditional blunt-end making to sticky-end making. We clarified that certain sticky ends can be made by KF in controlled digestion, and determined that the exact sticky-end length was 1~2 nt. Although the 2 nt overhang produced by KF is transient (5–10 min), our results showed that this is enough for ligation and cloning. Many polymerases have 3′-5′ or 5′-3′ exonuclease activities; consequently, it is possible that they can be used in cloning in the same way. Our article also provides a simple and quick method to find appropriate enzyme concentration and reaction time for different exonucleases.

Compared with RE sticky end, the sticky-end sequence made by exonuclease can be designed. This is more flexible for multi-fragment ligation. By carefully designing, multi-fragment directional cloning with the same exonuclease, such as KF, in one step is possible. This will also avoid buffer incompatibility problem of using different enzymes, and is more convenient than choosing different REs to make different sticky ends. By optimizing reaction conditions, insertion fragments, such as PCR products, can be also sticky-ended by KF, and be inserted into plasmid. In flexible cloning design strategy, this method can also combine with RE method, and the sticky end made by exonuclease and that made by RE can be ligated together. An RE that produces a 2 nt long sticky end is easy to find, such as *Nde*I, *Asn*I. A method of 2 nt overhangs ligation has also been discussed (Ranjan and Rajagopal 2010).

For suitable sticky ends made by KF, the controlled reaction condition is important. For example, elongated reaction time resulted in disappearance of the sticky-end bands and the complete cleavage of duplex (figure 3). Other conditions such as KFs from different commercial sources may vary in quality and efficiency, and thus affect this method to some extent. Although condition determination needs some work, once the optimal condition is acquired, sticky ends of varied DNA substrates can be made conveniently. As we showed in this study, both linearized plasmid and insertion fragment can be prepared to be sticky end for large scale use in the cloning steps.

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