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A high-throughput and single-tube recombination of crude PCR products using a DNA polymerase inhibitor and type IIS restriction enzyme

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ABSTRACT

Type IIS restriction enzymes have been successfully used as “universal” restriction enzymes in DNA manipulations. We took a step further to develop a rapid technique for recombining DNA fragments, fully automatic single-tube recombination (FASTR), which enables multiple-fragment DNA recombination in a single step. Crude PCR products are directly mixed with both type IIS restriction endonuclease and DNA ligase to initiate a spontaneous and one-way recombination reaction. Highly efficient DNA recombination can be achieved by an inhibition of DNA polymerase with aphidicolin and a selective digestion of template DNAs by DpnI, a restriction enzyme to digest hemi-methylated DNA in the reaction solution; thereby the entire procedure takes less than 15 min. Owing to its simplicity, efficiency and rapidity, one-step FASTR can be applied to a wide range of DNA manipulations including those involving high-throughput applications where significant reduction in time and cost is expected.

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1. Introduction

There have been various approaches to making cloning simpler or faster: instead of using classical restriction enzymes and DNA ligase, DNA recombination systems from different organisms have been used to manipulate plasmid DNA (Hartley et al., 2000; Liu et al., 1998). These techniques have made some repetitious gene clonings easier, but because they are based on DNA recombination, special sequences must accompany the gene to be cloned and the destination vector. These sequences are not removed during the recombination reaction, which is problematic when one tries to fuse one gene to another with a defined linker sequence. Another problem is that if a destination vector is not available for a certain application, one has to make it through conventional plasmid construction. Thus, recombination-based cloning techniques can lack to some degree the flexibility of classical cloning technology.

Type IIS restriction enzymes digest DNA sequence outside of their recognition sequence (Kleid et al., 1976; Takemori et al., 2002). With recognition sequences on primers, type IIS enzymes can be utilized as a universal restriction enzyme for the cloning of PCR products (Podhajska and Szybalski, 1985). We refocused on this classic enzyme because of its versatility and potential for a fast spontaneous DNA recombination reaction. With an inhibition of residual thermostable DNA polymerase activity and selective digestion of template DNAs, we are now able to recombine multiple DNA fragments directly from crude PCR solution in a single tube within 15 min.

2. Materials and methods

2.1. FASTR reaction

PCR was carried out using a high-fidelity thermostable DNA polymerase, KOD-plus (Toyobo, Osaka, Japan) according to the manufacturer’s protocol. Briefly, the reaction buffer contained 1× KOD-plus buffer, 2 mM dNTPs, 25 mM $MgSO_4$, 1 unit of KOD-plus, 25 pmol each of forward/reverse primers, and 50 ng each of template plasmids, in a total volume of 50 μl. The template plasmids for mSECFP (Matsuda et al., 2008) and mVenus (Venus (Nagai et al., 2002) with A206K mutations (Zacharias et al., 2002)) were in a pcDNA3.0 vector (Invitrogen, CA, USA), and mCherry (Shu et al., 2006) was in a plant expression vector. pRSET B vector (Invitrogen, CA, USA) was modified by site-directed mutagenesis to delete an LguI site at nucleotide position 2830. The PCR conditions were as follows: 94 °C/2 min (94 °C/15 s, 60 °C/30 s, 68 °C/90 s), 35 cycles, 68 °C/5 min. One microliter of the PCR solution from each sample was transferred to a mixture containing the following reagents in a total volume of 20 μl: 17 mM Tris–acetate, 25 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 5 mM magnesium acetate, 33 mM potassium acetate, 5 mM ATP, 5 mM DTT, 63 μg/ml BSA, 5 U of LguI

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(Fermentas, ON, Canada), 400 U of T4 DNA ligase (NEB, MA, USA), 20 U of DpnI (NEB, MA, USA), and 5 mM aphidicolin (Calbiochem, CA, USA). The mixture was incubated without agitation at room temperature for various lengths of time. After incubation, 5 μl of the mixture was used to transform *Escherichia coli* (JM109 DE3, Promega, WI, USA). The sequences of the primers used in the FASTR reaction were as follows: pRSET forward primer: GCTACT-GCTCTTCGTGCGCTGGTACCATGGAATTCGAA, pRSET reverse primer: CTGATAGCTCTTCTCACGTACAGATCCCGACCCATTTG, GFP-variants forward primer: GCTAGCTCTTCAGTGATGGTGAGCAAGGGCGA and GFP-variants reverse primer: CTAGGCTCTTCTGCACTTG-TACAGCTCGTCCATGC. For multiple-fragment FASTR the following sets of primers were used to amplify the inserts: GFP-variants forward 1: GCTAGCTCTTCAGTGATGGTGAGCAAGGGCGA, GFP-variants reverse 1: CTAGGCTCTTCTACCCTTGTACAGCTCGTCCATGC, GFP-variants forward 2: GCTAGCTCTTCAGGTATGGTGAGCAAGGGCGA, GFP-variants reverse 2: CTAGGCTCTTCTGCACTTGTACAGCTCGTC-CATGC.

2.2. Fluorescent colony imaging

For the single-color imaging of mSECFP-expressed *E. coli* colonies, an LAS-1000plus image analyzer (Fujifilm, Tokyo, Japan) was used to take fluorescent images. For multicolor imaging, a hand-made device equipped with a Mercury lamp, interference filters, optical fiber cables, and video CCD camera (WAT-120N, Watec, Yamagata, Japan) was used. The filter combination was as follows: 440/21 excitation and 480/30 emission for mSECFP, 500/24 excitation and 535/26 emission for mVenus and 540/30 excitation and 575LP for mCherry. All images were acquired using ImageJ software. MetaMorph (Molecular Devices, CA, USA) was used for image analysis.

2.3. Determination of FRET efficiency

Venus/T-sapphire fusion protein was mixed with 0.05% trypsin–EDTA (Gibco, CA, USA) in PBS and incubated at 37 °C for 1 h. The samples with and without trypsin were analyzed by F-2500 fluorescence spectrophotometer (Hitachi, Tokyo, Japan). The peak values of the donor protein (T-sapphire) before and after the trypsin treatment were used to calculate the FRET efficiency, according to the following equation:

$$E = 1 - \frac{F_{DA}}{F_A}$$

where E is the FRET efficiency of the donor/acceptor pair, F_{DA} is the fluorescence intensity of the donor in the presence of the acceptor (before trypsin treatment), and F_A is the fluorescence intensity of the donor in the absence of the acceptor (after trypsin treatment).

3. Results

3.1. The concept of the FASTR reaction

The DNA cleavage site of type IIS endonucleases is at a distance from their DNA recognition site, thus these enzymes create DNA overhangs of any nucleotide sequence (Szybalski et al., 1991). This feature is advantageous for DNA manipulation in many ways. In carefully designed plasmid constructions, the DNA recognition site for type IIS enzymes can be removed, allowing the ligated final product to be tolerant of the endonuclease that created the overhangs (Lebedenko et al., 1991). This method is more flexible than those using conventional type II endonucleases, because the joint sequence can be designed freely. Another advantage is that the cleavage site does not have to be palindromic, thus preventing undesired homo-ligation between inserts or vectors. We reasoned that, with these features, it might be possible to have both ligation and endonuclease digestion occur simultaneously in the same tube.

As shown in Fig. 1A, all the terminals of the DNA fragments in FASTR carry cleavage sites for type IIS endonuclease followed by recognition sites for the endonuclease. The recognition sites are always outside of the cleavage sites, so after digestion by type IIS endonuclease, no recognition site remains on the DNA fragment. Because both the endonuclease and the ligase are present in the reaction mixture, equilibrium between digestion and ligation is achieved, as long as the ligated product carries the recognition site for the endonuclease. Once ligation between the designated DNA fragments is completed, there is no more digestion even in the presence of the endonuclease, because of the loss of the recognition site. It is this directional reaction that drives the designated products out of the equilibrium, enabling the recombination reaction in a single step (Fig. 1C). The sequence of the cleavage site is independent of the recognition site, allowing the terminus of each DNA fragment to have a specific sticky end for a specific partner fragment. If a three-nucleotide sticky end is used for FASTR, one can theoretically use 4^3, or 64, specific sticky ends, enabling multiple-fragment ligations (Fig. 1B).

3.2. FASTR reaction using gel-purified DNA fragments

To demonstrate this single-step recombination reaction, we amplified both the insert and vector fragments by PCR using pairs of primers with specific cleavage and recognition sites on each end. The amplified fragments were gel-extracted and mixed with type IIS endonuclease (LguI) and T4 DNA ligase. After 2 h of incubation at room temperature, 5 μl of the reaction mixture was used directly to transform chemically competent *E. coli*. To our surprise, eight samples from eight colonies gave positive results for both single- and double-fragment insertions (Fig. 2A). DNA sequencing confirmed that the sequences of the joint region were exactly as designed, verifying the validity of the FASTR reaction (Fig. 2B).

3.3. FASTR reaction using crude PCR products

Encouraged by the result, we thought it might be possible to recombine crude PCR products in a single step. In our early attempts to use this single-tube method, only few positive colonies were obtained (data not shown). We reasoned that the residual activity of the thermostable DNA polymerase would convert the sticky ends to blunt ends, giving rise to self-ligation of the vector plasmid. If so, a DNA polymerase inhibitor might help to reduce the unwanted self-ligation.

Aphidicolin has been known to inhibit the alpha subtype of DNA polymerase in eukaryotes, as well as some types of archaeal DNA polymerases (Ikegami et al., 1978; Klimczak et al., 1985). The KOD-plus DNA polymerase was derived from archaeal *Pyrococcus kodakaraensis* (Takagi et al., 1997). We therefore hypothesized that aphidicolin could inhibit the residual activity of the KOD-plus DNA polymerase present in the PCR product solution, thus allowing a higher recombination rate in the following FASTR reaction. To test the hypothesis, aphidicolin at a final concentration of 2.5 mM was added to the PCR product solution, and 1 μl each of the insert (cyan fluorescent protein, mSECFP) and vector (pRSET B) with the LguI site mutated) solutions were mixed with LguI restriction endonuclease and T4 DNA ligase. DpnI was also included in the reaction to digest the residual template DNA. After vari-

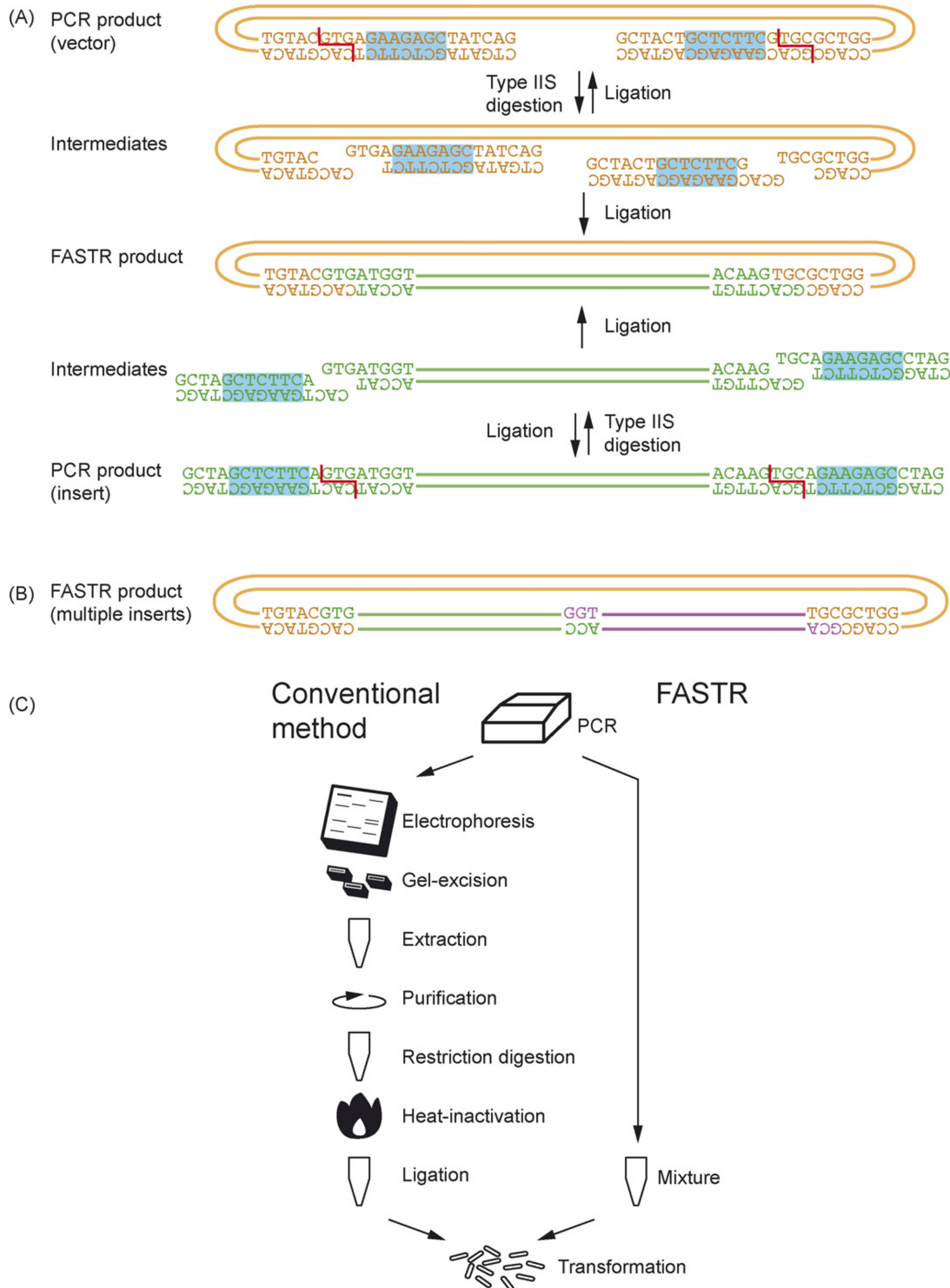

Fig. 1. Schematic representation of the FASTR reactions. (A) PCR products (top and bottom) are treated with both type IIS endonuclease and DNA ligase to induce equilibrium reactions of digestion and ligation. Once the FASTR product is assembled (middle), the reaction stops, because the final product lacks the recognition site for the endonuclease. Blue boxes represent the recognition site for type IIS endonuclease and red lines are the cleavage site of the endonuclease. Vector DNA is in orange and insert DNA is in green. (B) An example of FASTR product with multiple inserts. The inserts are indicated in green and purple. (C) Outline of the FASTR protocol. The entire procedure of FASTR is carried out in a single tube and takes less than 15 min.

ous incubation times at room temperature, JM109 DE3 competent cells were transformed with 5 μl of the FASTR reactant. With 15 min of FASTR reaction at room temperature, more than half of the colonies were fluorescent upon illumination of the plate by 470 nm blue LED. The recombination efficiency reached close to 80% when the incubation time was extended to 2 h (Fig. 2C and D).

3.4. *Multiple DNA fragment assembly by FASTR reactions*

We next examined the possibility of performing multiple-fragment FASTR with the crude PCR products. The procedures were essentially the same as for single-fragment FASTR, except that the joint sequences were designed so that the insertion fragments would be fused in the correct direction and order

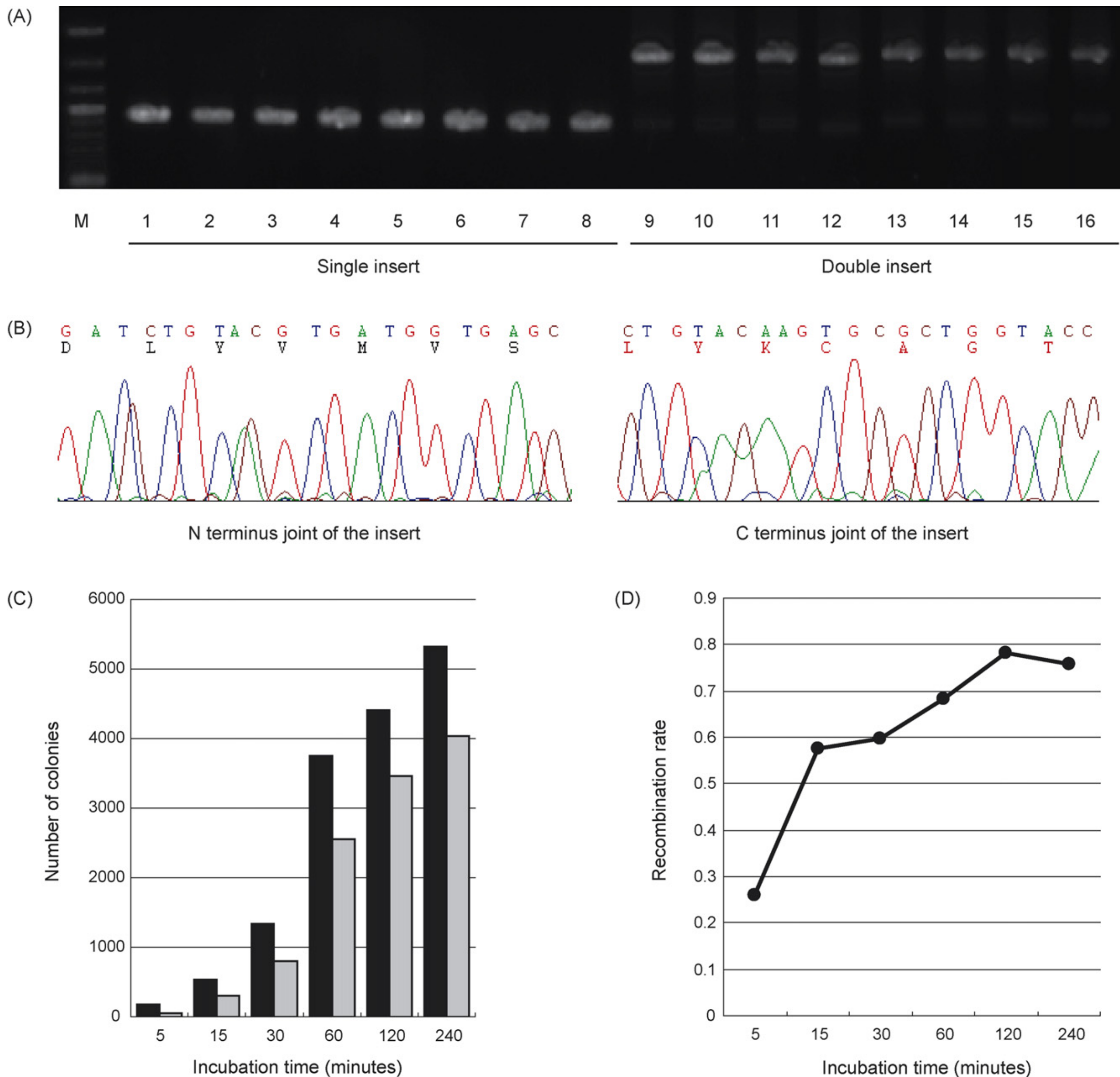

Fig. 2. High recombination rate of FASTR. *E. coli* colonies were obtained by the transformation of FASTR products. The colonies were subjected to colony PCR for insert analysis. (A) Eight randomly picked colonies were positive for single and double insertions. (B) The joints between the vector and insert were DNA sequenced to confirm the validity of the method. The FASTR reaction was carried out for various lengths of time. The number of transformed colonies reached near-plateau at 60 min (C); however, the recombination rate changed little after 15 min (D). Black bars represent the total number of colonies, and gray bars represent the number of fluorescent colonies. The recombination rate was calculated by dividing the number of fluorescent colonies by the total number of colonies.

(Fig. 1B). We amplified the insert fragments of cyan fluorescent protein (mSECFP), yellow fluorescent protein (mVenus), and red fluorescent protein (mCherry) along with the vector fragment of pRSET B. The fragments were then mixed to assemble pRSET–mSECFP–mVenus and pRSET–mSECFP–mVenus–mCherry constructs. The FASTR reaction was performed at room temperature for 1 h. We confirmed that close to half of the fluorescent colonies were double positive for mSECFP and mVenus fluorescence in pRSET–mSECFP–mVenus construct, and around 20% were triple positive for mSECFP, mVenus, and mCherry fluorescence in pRSET–mSECFP–mVenus–mCherry sample (Table 1 and Fig. 3A). These colonies were further analyzed by colony PCR to confirm the inserts (Fig. 3B).

3.5. *High-throughput screening of a high-performance FRET probe by FASTR*

To demonstrate the feasibility of FASTR in a practical application, we constructed a GFP-based Förster resonance energy transfer

Table 1

Insert	Number of positive colonies	Number of fluorescent colonies	Recombination rate (%)
CFP+Venus	517	1050	49.24
CFP+Venus+mCherry	50	242	20.66

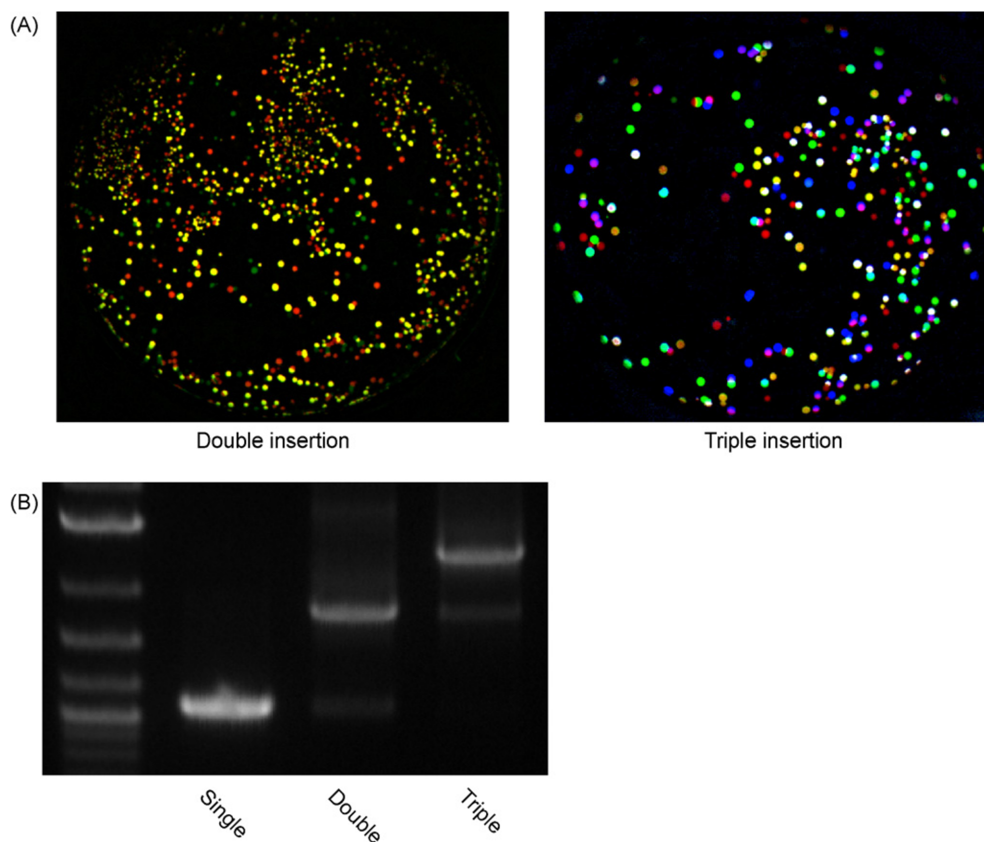

Fig. 3. Double and triple insertion by FASTR. (A) Fluorescent images of colonies expressing mSECFP, Venus, and/or mCherry were taken with multiple filter sets. The images from different color channels were overlaid and pseudocolored. Yellow colonies in the double insertion and white colonies in the triple insertion images are double and triple positives, respectively. (B) Insert lengths of the positive colonies selected by fluorescence were confirmed by colony PCR.

(FRET) pair with various linker regions to maximize FRET efficiency. FRET-based GFP probes have been widely used in biological studies as a genetically encoded molecular sensor (Piston and Kremers, 2007). One obstacle to the technology is that FRET efficiency changes dramatically with very small changes in the distance and relative orientation between fluorophores (Lakowicz, 2006). The sensitivity of a FRET pair is so acute that even a single amino-acid insertion or deletion in the linker region is enough to affect the FRET efficiency. Thus, the construction of a FRET pair requires laborious linker optimization by trial and error (Nagai and Miyawaki, 2004).

Here, we used FASTR to fuse a UV-excitable green fluorescent protein (T-Sapphire) (Zapata-Hommer and Griesbeck, 2003) to Venus, with various linker lengths between them (Fig. 4A). Thirteen reverse primers were designed for C-terminal deletion mutants of Venus, and seven forward primers for N-terminal T-Sapphire deletions. A total of 20 deletion mutants were PCR-amplified. Then 1 μl of the crude PCR products from each sample were mixed in one tube along with FASTR reagents. The FASTR reaction should recombine two inserts and a vector with correct order but in random combinations, giving rise to a total of 91 mutant combinations. After 2 h of incubation at room temperature, JM109 competent cells were transformed with 10 μl of the reactant. *E. coli* plates were screened for yellowish fluorescent colonies using a 405 nm light source. DNA sequencing of the linker region and spectroscopic analysis of the expressed proteins identified the clones with high FRET efficiency (Fig. 4B). The FRET efficiency of the best clone we obtained was determined to be 67% by cleaving the linker region (Fig. 4C), demonstrating that FASTR is an ideal method for creating a large number of multiple-fragment constructs for high-throughput screening.

4. Discussion

Although cloning techniques with type IIS restriction endonucleases and DNA ligases have been widely used, the endonuclease-mediated single-step recombination in our method is much faster than previous approaches. As far as we know, this is the first report which describes the spontaneous and directional single-tube recombination with a type IIS restriction endonuclease and DNA ligase. The reaction allows highly efficient DNA recombination, while keeping a high flexibility of class IIS restriction enzymes. Another advantage of our approach is the ability to directly recombine crude PCR products. This was made possible by adding an archeal DNA polymerase inhibitor to the FASTR reaction. Although we have not tried a monoclonal antibody against the polymerase, it might also be used to inactivate the residual polymerase activity, as it is for hot-start PCR (Kellogg et al., 1994). DpnI was also included in the reaction to selectively digest the bacterially expressed template DNA in the crude PCR products.

We primarily focused our efforts on improving the rapidity of the method, because we thought that raising the speed limit for DNA recombination would enhance research in many fields, especially ones involving high-throughput analysis. One such field is the development of GFP-based FRET probes, which are now indispensable in the biological sciences. A typical FRET probe has fluorescent donor and acceptor domains along with a sensor domain to detect biological signals. High FRET efficiency is achieved only when the distance between fluorophores is comparable to the Förster distance, and the relative dipole moments of the fluorophores are parallel to each other. The distance between fluorophores is largely affected by the linker sequences between

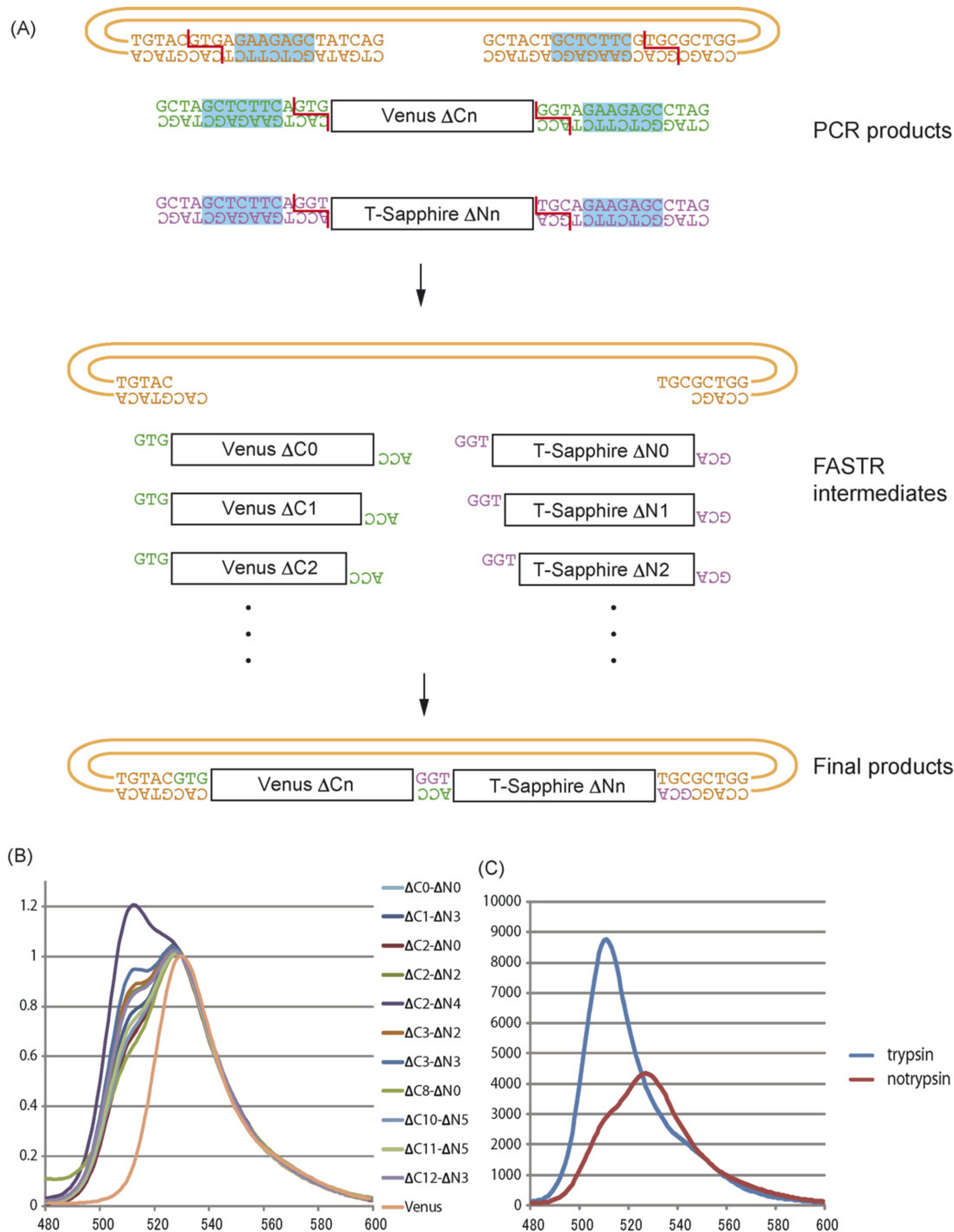

Fig. 4. Optimization of a GFP-based FRET pair. (A) A schematic representation of FRET pair optimization by FASTR. GFP variants with various lengths of linker regions are PCR-amplified (top). FASTR intermediates are indicated in the middle panel. The final products are represented in the bottom panel. An N-terminal deletion is indicated as ΔN1 and a C-terminal deletion as ΔC1. '*n*' deletions are represented as ΔN*n* or ΔC*n*. (B) Fluorescence spectra of Venus (511 nm) and T-Sapphire (527 nm) fusion proteins with various linker regions. The spectra were obtained by 395 nm illumination and fluorescence scanning with a 5 nm slit. The curves were normalized to the fluorescence intensity at 530 nm. (C) Venus-T-sapphire (ΔC2–ΔN0) fusion protein was cleaved into separate fluorophores by trypsin. The FRET efficiency of the pair was calculated by fluorescence recovery of the donor protein (T-sapphire).

the domains, and the relative orientation of the fluorophores can be changed by using circular permutations of the fluorescent proteins (Nagai et al., 2004). Since it is very difficult to predict the structural state of the fluorophores by calculation, one has to optimize these parameters through empirical methods. FASTR technology can contribute to the development of such probes, because it can assemble multiple domains of FRET probes simultaneously, saving the time and cost of constructing numerous combinations of linkers and domains to obtain a favorable FRET signal.

A possible drawback of FASTR methodology would be the requirement of primers for each FASTR reaction. Although the cost and availability of synthetic oligonucleotides have been greatly improved, the requirement might be an obstacle to some researchers. Another drawback would be the imcompatibility of FASTR products with conventional restriction enzymes; the liga-

tion site of the FASTR products cannot be recut with conventional restriction enzymes. We recommend another round of FASTR reaction for an additional plasmid modification, and colony PCR for insert check.

FASTR technology is among the quickest in the latest generation of recombinant technologies, while it achieves the high efficiency of recombination-based methods and the flexibility of conventional cloning. These advantages should be applicable to most DNA manipulation procedures, including high-throughput DNA manipulations, where significant savings in time and cost are expected.

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