Lego DNA assembling, a simple in vitro method for constructing DNA molecule

Abstract

Digestion-ligation based and recombination based methods for constructing recombinant DNA are the basic techniques in molecular biology and thus built one of the foundations for the modern life sciences. Here we describe a new strategy that can radically simplify some of the same task. The lego DNA assembling, based on strand annealing, allows in vitro assembly of multi DNA fragments in one step with precise junctions and excludes the need for any enzyme. As a proof of concept, we rapidly constructed plasmids from 4, 6 and 8 fragments with very high efficiencies (100%). And we found this method a powerful tool for synthetic biology, constructing a partial isoprene biosynthesis pathway (consisting of four genes) in 2 days. We also assembled a customized expression vector to show its modularity.

Introduction

Modifying organisms at the molecular level is the main base for modern biotechnology. In most cases of the DNA manipulation part, people need to incorporate two or more DNA fragments together into a relative complex and functional structure. For this task, many methods have been invented in the last 40 years.

When only two fragments are involved, the traditional restriction-enzyme-based assembly methods are most commonly used[1]. Other methods based on PCR, including splicing by overlap extension (SOEing)[2], PCR-induced (ligase-free) subcloning[3], Ligation-independent cloning of PCR products (LIC-PCR)[4], recombinant circle pcr[5] and methods based on site-specific recombination in vitro or in vivo, e.g., the Univector Plasmid-fusion System[6], Gateway system (Invitrogen), recombinational cloning[7] and homologous recombination based cloning methods in yeast[8-13] or in *E. coli*[14] are used in certain situations.

Recently, genetic engineering has moved beyond this traditional single gene cloning. For example, in metabolic engineering, biosynthetic pathways with up to tens of steps are often set up in heterologous organisms to obtain products of interest[15-17]; in synthetic biology, complex genetic circuits are constructed to achieve desired functionalities[18-19]. Consequently, assemblies of multi genetic elements are now widely required. The conventional strategy to do this is repeating the multiple-step cloning which includes PCR amplification, restriction digestion, in vitro ligation and transformation. As the whole process is carried out in a sequential manner, it is very time and labor consuming. And as the number of fragments and the length of the recombinant DNA increase, it can be impossible to find unique restriction sites. To

address these limitations, two new methods able to assemble multiple DNA fragments in one single cycle and sequence–independent were recently reported. Both of them were based on homologous recombination. Sequence and ligation-independent cloning (SLIC) used T4 DNA polymerase and RecA in vitro to mimic the RecA-mediated recombination system of *Escherichia coli*[20]. DNA assembler directly used the in vivo homologous recombination in *Saccharomyces cerevisiae*[21]. Besides Ordered Gene Assembly in *B.subtilis* (OGAB) using restriction endonuclease SfiI and T4 DNA Ligase can also assemble up to 6 fragments simultaneously by exploiting the highly effective DNA uptake and plasmid establishment in *B.subtilis*[22]. Those methods either were limited to specific hosts or need enzymes. And faulty recombinations were observed for both in vivo and in vitro recombination based methods when the numbers of fragments increased or repeat sequences existed.

Here we report a much simpler strategy for DNA fragments assembly called lego DNA assembling. As shown in **Fig. 1** successive substrate fragments (SFs, analogues to the bricks of a lego cycle) that designed to have long overlaps with each other were mixed, denatured and annealed. As those overlaps are very long and can cover about 1/3 to 2/3 of each SF, there are good chances for inter-fragment hybridization. Then a circle plasmid can form and be ready for transformation.

As a proof of concept, we applied this method to construct plasmids from 4, 6, 8 SFs respectively, and assemble a 4-gene partial isoprene-biosynthesis pathway and a customized expression vector. We found that the unique mechanism of lego DNA assembling gave it an outstanding performance.

Results

SFs preparation

SFs were prepared by simple PCR, overlap extension PCR[2], long tailed primer PCR, ligation or chemical synthesis in this article. It is also reasonable to think that fragments prepared by other means will also work as long as they obey to the sequence principle shown in **Fig. 1**.

Annealing and construction

The core step of our method, annealing, was carried out simply with a beaker. Briefly speaking, approximately equal quantity of SFs were mixed and sealed in a centrifuge tube and then submerged in a beaker of boiled water, left in room temperature till it cool down. **Fig. 2** shows the annealing result of our 4-SF construction. The weakening of the 4 SFs bands and the appearance of new bands indicated that inter-fragment hybridization occurred during the annealing. Although the band of cycle molecules couldn't be discerned, our following experiments showed that there were sufficient complete molecules for very fine transformation results. The annealed mixture was directly used for transforming *Escherichia coli* chemical competent cells. An aliquot of the mixture without annealing treatment was used as negative control.

Construction of a 4-SF plasmid

SF 2, 3, 4 were prepared by overlap extension PCR, while SF1 was amplified directly from pET28a. Three antibiotic (ampicillin, kanamycin, chloramphenicol) resistance genes and a replication region were coded by them in a pattern shown in **Fig. 3a**. After mixing and annealing, 5 microliters reaction was used to transform DH5a chemical competent cells by standard method. Aliquots of the transformation reaction were

spread on LB plates containing the three antibiotics respectively. 100 resulting colonies randomly picked from each plate were screened by colony PCR. Then the positives were subjected to restriction mapping and antibiotic tests to see if they have correct structures and gene functions. On Cm and Amp plates about 51,000 and 73,000 colonies/ microgram SF DNA were obtained. 98 out of 100 passed the colony PCR screening. 100% of the passed ones (10/10) exhibited the expected restriction map (**supplementary Fig. 1a**) and showed all three antibiotic resistances. On Kan plate, about 161,000 colonies/ microgram SF DNA were got, 16% showed correct results in colony PCR screening, restriction mapping and antibiotic tests. In contrast, the negative control gave 0, 0 and 265 colonies on Cm Amp and Kan plates respectively. The false positives from Kan plates were investigated by restriction mapping and identified to be pET 28a, the PCR template for SF1 (data not shown). Apparently some circular plasmids escaped linearization and *DpnI* digestion. But why they could cause so many background colonies is still unclear. High fidelity of lego DNA assembling was suggested since the PCR screening positives always showed all three antibiotic resistance activities.

Construction of a 6-SF plasmid

To show the scalability of this method, we constructed a 6-SF plasmid based on the 4-SF plasmid by replacing SF1 with SF 5, 6, 7. An ura3 selection marker and a 2μ replication origin for maintenance in *Saccharomyces cerevisiae* were thus added, making this plasmid a shuttle one (**Fig. 3b**). SF 5, 7 were prepared by overlap extension PCR; SF6 was amplified directly from pDR195[23]. As the number of fragments increased, fewer colonies were yielded: 17,000, 16,000 and 26,000 colonies on Cm, Amp and Kan plates from 1 microgram SF DNA. 98%, 100% and 14% could be screened out by colony PCR.

All of the positives showed correct results in restriction mapping (**supplementary Fig. 1b**), and antibiotic tests.

Construction of an 8-SF plasmid

To further demonstrate the capacity and flexibility of lego DNA assembling, we reconstructed the first plasmid from 8 SFs. 4 of the 8 overlaps were shortened to 50 nt. All 8 SFs were prepared by tailed primer PCR. Sequences of 25 nt were added to 5' ends of primers to generate the 50-nt overlaps on one end of each SF (**Fig. 3c**). A mixture of 1 microgram of each SF yielded 40, 144, and 96 colonies on Cm, Amp and Kan plates. 80%, 72% and 50% of them were confirmed to be correctly constructed by PCR screening, restriction mapping (**supplementary Fig. 1c**) and antibiotic tests.

During the cooling process of the fragments mixture, the longer overlaps should anneal first leaving the shorter 50-nt overlaps still free as single strands. As a result, four intermediates with 50-nt cohesive ends would form and be further combined into a cycle mediated by those cohesive ends as the mixture get cooler. In this way, it bears some resemblance to an existing cloning strategy which is based on the phenomenon: relatively longer cohesive ends (usually more than 12nt, compared with the 2- or 4-nt ends created in restriction-ligation cloning) can mediate more stable joint between insert fragment and vector, making the ligation treatment unnecessary[24]. Such strategy has many variations according to how the cohesive ends are created, e.g., using exonuclease (LIC-PCR[4] and SLIC[20]), using mixed PCR (Enzyme-free cloning[25] and SLIC [20]), using terminator primers PCR[26], or using incomplete PCR (SLIC)[20]. Compared with them, our method is simpler to practice and can join more fragments together.

Construction of a 4-gene partial isoprene-biosynthesis pathway

By lego DNA assembling, we finished this construction within 2 days, including the time for SFs preparation. The vector backbone and genes encoding the last 4 enzymes of *S. cerevisiae* MVA pathway, *ERG12* (coding for mevalonate kinase), *ERG8* (coding for phosphomevalonate kinase), *ERG19* (coding for mevalonate pyrophosphate decarboxylase) and *idi* (coding for isopentenyl pyrophosphate isomerase), were first constructed in 6 SFs by simple PCR or overlap extension PCR and then combined together by lego DNA assembling. Three RBS-containing sequences of about 45bp each were introduced by tailed primers during the PCR to separate and organize all 4 genes into a single operon (**Fig. 3d**). Out of 30 picked colonies, 16 were screened out as positive. All of them had the correct map (**supplementary Fig. 1d**). One was further sequenced for the operon area. No mutation was found in this 5.1 kb area.

The resulting plasmid, pTRICLow, was cotransformed with pACY-Isp4 (coding for isoprene synthase, constructed previously in our lab by traditional cloning) into *E. coli* BL21 (DE3). The constructed strain was cultured in a McCartney bottle for isoprene biosynthesis from mevalonate (MVA). GC-MS headspace analysis was carried out to detect the isoprene production. Culture not treated with MVA or culture of strain containing empty plasmids was used as negative control. The isoprene peak was identified by both retention time and mass spectrometry. As shown in **Fig. 4** the strain harboring the partial isoprene-biosynthesis pathway gave out a prominent isoprene peak when MVA was added. As *E. coli* can also produce low concentration of dimethylallylpyrophosphate, the substrate for isoprene synthase, by native DXP pathway, a much smaller peak was also observed for the culture without MVA treatment (**Fig. 4**, negative control 1).

We also demonstrated that BL21 (DE3) could be transformed by the annealed mixture directly and similar efficiency was observed to that of DH5α.

Construction of a customized *Bacillus subtilis* expression vector

A vector is typically consisted of one or two replication origins, selectable markers, and multiple cloning sites. Other genetic elements such as promoter, terminator, expression regulatory element, reporter gene, targeting sequence can also be included. Till now, people have discovered and developed lot kinds of elements for vector construction which can endow the vector with different functional traits, e.g., host range, copy number, maintenance stability, temperature sensitivity, screening method, induction method, expression intensity, and product location in the cell. There are almost numerous kinds of possible combination of those genetic elements. On the other hand, existing vectors are usually limited and can't exactly meet all of our needs for a specific task. For example, in biosynthesis study, we may be satisfied with the stability and expression intensity of the commonly used commercial vectors but prefer a less costly induction method for scaling up our fermentation. So it is very desirable if we can pick every element we need and assemble them into a vector right before using. Similar to playing a lego game, we can get a great diversity of vectors from collections of genetic bricks by lego DNA assembling. The whole process can be finished in less than 2 days.

To demonstrate this, we constructed a shuttle vector for *E. coli* and *B. subtilis* containing 5 elements. A 3 kb long replication region from theta-replicating plasmid pBS72 provided the vector with full structural stability in *B. subtilis* [27-28]. A DNA segment from vector pNZ8901 containing chloramphenicol acetyltransferase gene and *spaS* promoter provided a selectable marker and the subtilin induction property[29]. Amp resistance gene

and replication origin of pBR322 made the vector can be maintained and selected in *E. coli* (**Fig. 3e**). Four SFs were first prepared by chemical synthesis (3SF3 and 3SF4) and overlap extension PCR (3SF1 and 3SF2), and then mixed and annealed.

E. coli chemically competent cells were transformed with the annealed reaction and plated on Amp plates. 10 colonies were picked. All of them passed the colony PCR screen and showed correct restriction map (**supplementary Fig. 1e**). One was further sequenced. No mutation was found.

Discussion

Lego DNA assembling got its name for two reasons. First, its mechanism is similar to assembling a lego cycle. Second, it owns great modularity like the lego game does, namely, we can rapidly assemble a customized structure by choosing desired component parts from collections. It is easy to use, doesn't require special equipment or any enzyme. And it constructs DNA in vitro, so is not limited to particular host like the in vivo methods.

Lego DNA assembling depends on the recognition between long DNA sequences. The whole sequence of the final molecule takes part in it. In all previous methods, no matter homologous recombination methods or sticky ends methods, only short sequences (usually no longer than 50 bp) take part in the recognition between two adjacent fragments. Firstly, this difference makes lego DNA assembling more robust. SFs don't need to be purified or modified by any enzyme; simple chemical transformation is enough for getting sufficient colonies. Secondly and more importantly, unlike previous methods, lego DNA assembling is not affected by repeat sequences that might exist in the final structure. For example, SLIC is not suitable for constructing the plasmid pTRICLow.

Because in SLIC, the three RBS-containing sequences between the four genes would appear at the ends of the insert fragments, right in the areas that supposed to mediated the recombination. Another example is the plasmid pOKC2μUA. Constructing it by DNA Assembler in *S. cerevisiae* is questionable. Repeat sequences of 81bp, 65bp, and 55bp were found in this plasmid. They are even larger than the homologous areas for the planned recombinations, and thus may cause unwanted recombinations inside the structure such as deletion[21].

As in all our 5 constructions we succeeded in the first attempts, not much effort was made to optimize our protocol. It is likely with further improvement one could construct larger structure from more fragments. For example electroporation can be adopted to achieve higher transformation efficiency; protoplast and cosmid packaging can be used to facilitate larger DNA molecules taking up.

As demonstrated in this article, SFs can be prepared by many ways. Among them, chemical synthesis is the most promising one. The sequence information is much easier to get than the physical molecule. And the cost of long DNA synthesis is dropping rapidly[30]. In the near future, a combination of DNA synthesis and lego DNA assembling may make the DNA manipulation an extremely easy task, giving people more freedom and time for DNA designing.

Experimental Protocol

Bacterical strains

We used *E. coli* DH5α for all our lego DNA assemblings. BL21 (DE3) was used for gene expression.

Media, chemicals and enzymes.

We used Luria-Bertani broth for culturing all our strains including *E. coli and B. subtilis*. Agar (1.5%) was added to make solid medium. We used antibiotics as follows: ampicillin (50 μg ml^{-1}), kanamycin (50 μg ml^{-1}), chloramphenicol (30 μg ml^{-1} for *E. coli* and 20 μg ml^{-1} for *B. subtilis*). IPTG (0.5 mM) was added to induce the expression of the T7-promoted genes.

Pyrobest™ DNA polymerase from TAKARA was used for SFs preparation. EastTaq™ DNA polymerase from Transgen was used for colony PCR screening.

Mevalonate was prepared by the method of Campos[31] from mevanolactone (Sigma)

SFs preparation

Regular PCR was carried out according to standard protocols. Overlap extension PCR was performed by the method of Shevchuk[32], except that Pyrobest™ DNA polymerase was used. All plasmid templates were linearized before using by appropriate restriction enzymes. PCR products were treated with *DpnI* at 37^{o}C for 1 h to further digest the templates. Primers and templates for PCR and primer sequences are given in **Supplementary table 1 and 2.**

If relatively short overlaps were involved, like in the case of our 8-SF construction, PCR products were purified by E.Z.N.A.^{TM} Cycle-Pure kit (OMEGA) to exclude residual primer oligonucleotides which's lengths were comparable to that of the overlaps. In other cases, purification was omitted.

Lego DNA assembling

Prepared SFs were checked by agarose gel electrophoresis. Roughly equal molar amounts of SFs were mixed in a 1.5 ml polyethylene tube and sealed with Parafilm for water-proof.

The tube was then submerged in a beaker of boiled water, left in room temperature till it cool down. Putting the beaker in a 4^{o}C freezer can accelerate the cooling process without affecting the efficiency of assembling.

The reaction was transformed into chemically competent cells with standard heat shock method.

Restriction mapping

Plasmids were isolated from overnight cultures using E.Z.N.A. ™ Plasmid Mini Kit from OMEGA, and then subjected to restriction digestion. The reaction mixtures were loaded to 1% agarose gels to check the correct restriction digestion pattern by DNA electrophoresis. Restriction endonucleases used and their expected results are given in **Supplementary table 3**.

Functional analysis of the 4-gene partial isoprene-biosynthesis pathway

Colonies were picked into 15 ml LB liquid medium containing Amp and Cm, grown overnight. 0.5 ml fresh overnight cultures were diluted 100-fold and incubated at 37 °C to an OD 600 between 0.6 and 0.8, then treated with 0.5 mM IPTG for 2 hours at 30 °C to express the genes. The cultures together with 6 mM MVA were transferred into sterile McCartney bottles and incubated for another 12h at 30 °C. The rubber seals of the McCartney bottles were smeared with silicone grease so that they would not contact with and absorb the isoprene generated inside the bottle[33]. Both the aerobic and anaerobic incubation were done at 180 rpm on an orbital shaker.

For sampling the headspace, an 85 μm polyacrylate fiber was inserted into the McCartney bottle with the help of a manual holder system (Supelco Inc, USA), allowed to equilibrate

with the headspace volatiles for 30 min at 50^{o}C and then placed into the GC injector immediately.

GC-MS analyses were performed with an Agilent 5975C GC-MS System equipped with a DB-5ms column (50 m length, 0.25 mm ID, 0.25 mm film thickness, Agilent, Palo Alto, CA, USA). Experimental chromatographic conditions were as follows: injector set at 250^{o}C; Helium carrier gas at 1ml/min; oven temperature program: 1 min isotherm at 40^{o}C followed by a linear temperature increase of 5^{o}C min^{-1} up to 80^{o}C and then 25^{o}C min^{-1} to 300^{o}C held for 5min. MS scan conditions: source temperature 230^{o}C, interface temperature 300^{o}C, E energy 70 eV, mass scan range 2–150 amu.

The isoprene peak was identified by both retention time and mass spectrometry.

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Figure Legends

Figure 1 scheme of lego DNA assembling (take an 8-SF assembling for example). Mixture of SFs is denatured at 100^{o}C to free all single strands. Every SF is designed to have its 3'-half sequence overlapped with the 5'-half sequence of the next SF. And the 5'-half sequence of the first SF overlaps with the 3'-half sequence of the last SF. As a result, when the mixture cools down to room temperature, annealing between those sequences can assemble the single strands one after another into a cycle.

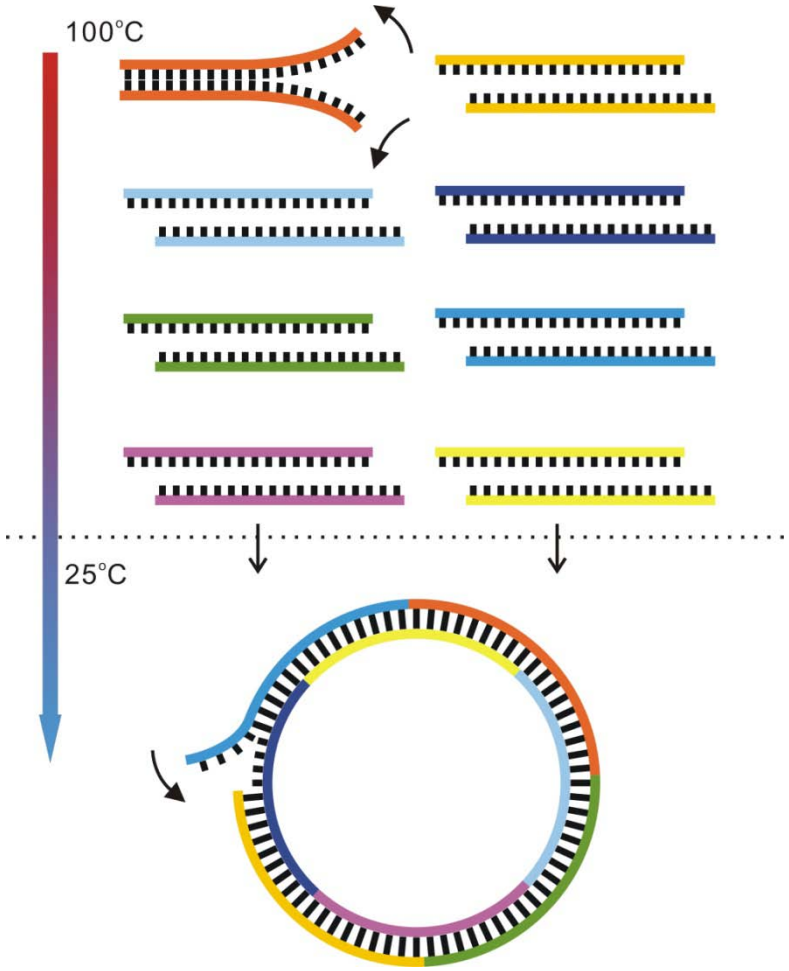
Figure 2 For constructing plasmid pOKCA, SF 1-4 were mixed (resulting M), annealed (resulting M&A) before transformation. Those six were added to agarose gel in a volume ratio of 1:1:1:1:4:4 then separated and visualized by ethidium bromide staining. From the comparison between M and M&A, we can find that, the bands corresponding to SF 1-4 weakened while at least two new visible bands emerged after the annealing treatment.

Figure 3 concentric circle schemes of the plasmids constructed in this article. The outer cycle shows the main genetic elements and the restriction map sites of each plasmid; the two inner cycles show the SFs used and the sequence ranges they covered.

Figure 4 GC-MS analysis of isoprene biosynthesis from MVA. Negative control 1: culture of strain containing empty plasmids, treated with MVA. Negative control 2: culture of strain containing pTRICLow and pACY- Isp4, not treated with MVA. (Mass spectrometric indentification of isoprene peak was shown in **supplementary Fig. 2**)

Figures

Figure 1:



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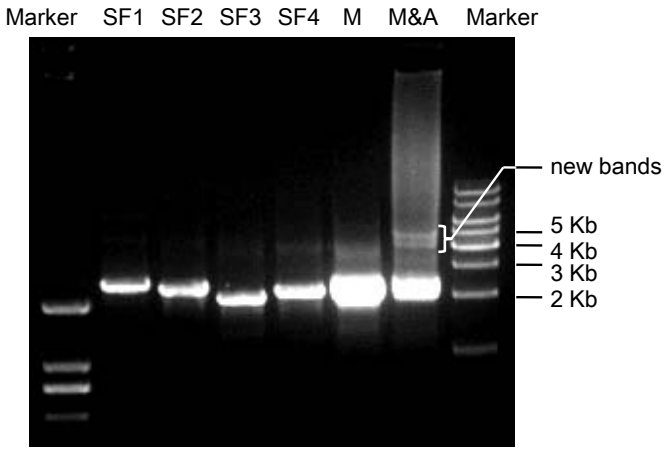

Figure 3:

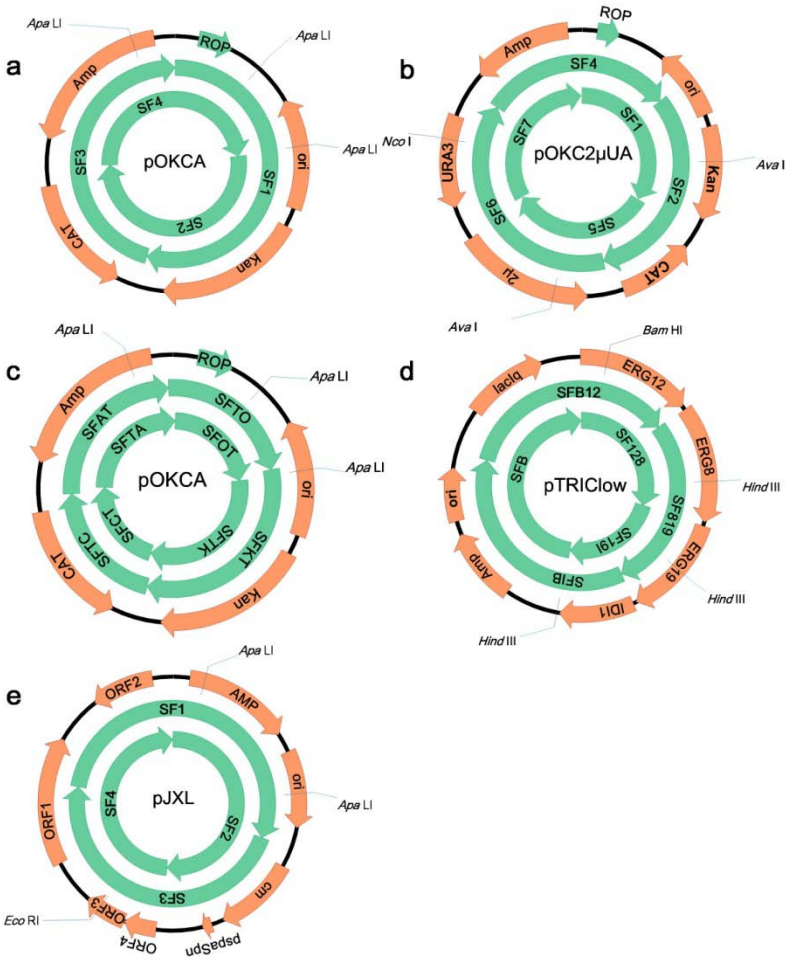
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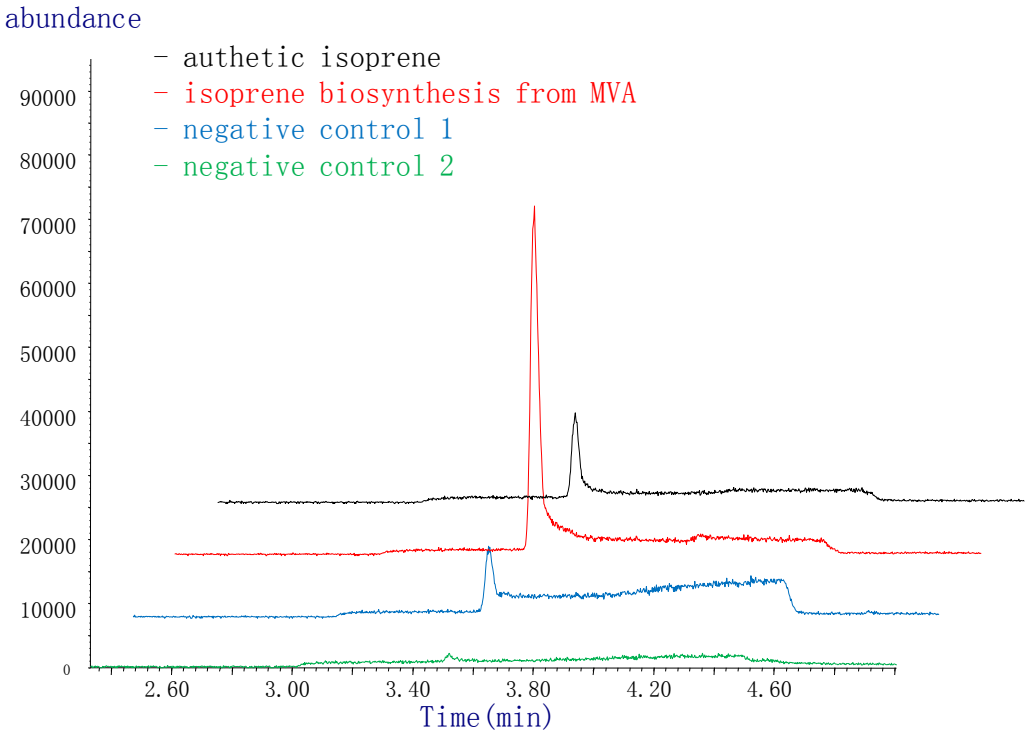
Figure 4: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