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Sequence-independent cloning methods for long DNA fragments applied to synthetic biology

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ABSTRACT

Simplified methods to assemble DNA fragments by independent cloning sequence have helped in the progress of synthetic biology, allowing some biotechnological processes to become economically viable by genetic improvement of microorganisms. We compared three methods of assembling six DNA fragments: PCR fusion-based, isothermal NEBuilder and circular polymerase extension cloning (CPEC). Double and triple fusion occurs directly with the PCR products using PCR fusion-based and NEBuilder methods. For multiple fragments the results showed higher efficiency by the CPEC method which allowed assembly of six fragments previously purified by agarose gel extraction, after a sequence of 20 annealing/extension cycles without any primer.

Some limitations of *in vitro* and *in vivo* approaches to DNA assembly, such as a lack of special restriction enzyme sites, homology sequences and the requirement for specific host cells can hinder their application [1].

When multiple DNA fragments need to be gathered, the challenge becomes even greater. In the last decades, cloning methods of multiple DNA fragments have been developed, such as the Gibson isothermal assembly method [2], the ligase cycling reaction (LCR) [3], sequence and ligase independent cloning (SLIC) [4], the PCR fusion-based method [5], and circular polymerase extension cloning (CPEC) [6].

Here we utilized three different methods aiming to assemble DNA fragments. Specifically, we used NEBuilder, a PCR fusion-based method and the CPEC method as tools for joining up to six DNA fragments, comparing the efficiency of each approach. We also verified the influence of purified fragments during DNA fusion.

Initially, the fragments GLSA (452 bp), GPD (817 bp), GLSB (3203 bp), GLSC (1708 bp), HYG (1043 bp) and GLSD (335 bp) were amplified individually. GLSA, GLSB, GLSC and GLSD were obtained from the region corresponding to the glucan synthase (GLS) gene of *Pleurotus ostreatus* (http://genome.jgi.doe.gov/cgi-bin/dispTranscript?db=PleosPC15_2&id=1052138&useCoords=1). GLSB and GLSC fragments are parts of the same GLS gene, but were divided into two smaller portions (3203 and 1708 bp). A pair of lower degenerate primers was designed to sequence and confirm a fragment of approximately 420 bp [7].

GPD corresponds to an active portion of the glyceraldehyde 3-phosphate dehydrogenase of the same fungus [8], and HYG fragment was extracted from the plasmid pAN7-1 corresponding to the hygromycin resistance gene [9].

PCR amplification: The reaction was prepared with 31 μL water, 10 μL Phusion HF buffer, 1 μL dNTP (10 mM), 2 μL of each primer (10 μM), 1.5 μL DMSO, 0.5 μL Phusion High-Fidelity DNA Polymerase (NEB) and a total of 2 μL of DNA. Thermocycling parameters started with a denaturation at 98 °C for 30 s, followed by 30 cycles of denaturation at 98 °C for 10 s, annealing for 15 s at 50, 60 or 63 °C (depending on the melting temperature − Tm), and extension at 72 °C, according to fragment size (30 s/kb). The composition of the amplification primers and the Tm are shown in Table 1.

Double fusion reactions between GLSA + GPD or HYG + GLSD and triple fusion between GLSA + GPD + GLSB or GLSC + HYG + GLSD were prepared with 29 μL of water, 10 μL of Phusion HF buffer, 1 μL dNTP (10 mM), 1.5 μL of DMSO, 0.5 μL

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Phusion High-Fidelity DNA Polymerase (NEB), 4 μL of DNA divided in equimolar amounts (0.05 pmol) for each fragment according to its concentration and 2 μL of each external primer (10 μM) added after the first 10 cycles. Thermocycling parameters were described in PCR amplification, with annealing at 50 °C.

The fusion was also tested by employing an enzymatic method similar to that of Gibson. The purified fragments or PCR products were incubated at 50 °C according to the recommendations of the kit HiFi DNA NEBuilder Assembly Master Mix. After the fusion reaction, an aliquot of 1 μL was diluted with 3 μL of water to a standard PCR reaction as described in PCR amplification, with annealing at 63 °C.

For the CPEC method, all the six fragments were individually amplified as described in PCR amplification, with annealing at 63 °C, cut from the gel and purified with a QIAquick Gel Extraction Kit. Reaction conditions and thermocycling parameters were programmed according to Quan and Tian [6]. The difference from the original method was that we did not use fragments that would result in a circular plasmid, but in a linear product that can be also used in transformation of fungi.

Fig. 1 shows all the fragments amplified by PCR, which overlap each other by about 19–29 bp. After amplification and assembly reaction all the fragments resulted in slightly larger size than those analyzed alone. It is because they include overlapping portions of the previous and subsequent primers. Besides that the external fragments GLSA and GLSD include ends to connect in pRS426 plasmid.

Sequencing analysis (data not shown) confirmed that the 420 bp fragment corresponds to a portion of the catalytic region (FKS) between GLSB and GLSC of the β-glucan synthase gene from *Pleurotus ostreatus* with 99% similarity [7].

Fig. 2 (Lane 2) shows the double fusion of the fragments GLSA (452 bp) and GPD (817 bp). Lane 4 shows the fusion of three fragments, GLSA (452 bp), GPD (817 bp) and GLSB (3203 bp). The largest fragments used in each assembly were shown in Lane 1 (GPD – 817 bp) and Lane 3 (GLSB – 3203 bp) and were considered as size reference.

Lane 2 of Fig. 3 shows the fusion of two fragments (HYG – 1043 bp + GLSD – 335 bp) and Lane 4 shows the fusion of three fragments (GLSC – 1708 bp, HYG – 1043 bp and GLSD – 335 bp). The largest fragment used in each assembly is shown in Lane 1 (HYG – 1043 bp) and Lane 3 (GLSC – 1708 bp) and were considered as size reference.

The fragments in Lanes 2 and 4 (Figs. 2 and 3) demonstrate an increase of the molar mass after the PCR fusion-based reaction, indicating the incorporation of pairs of bases in PCR products. After

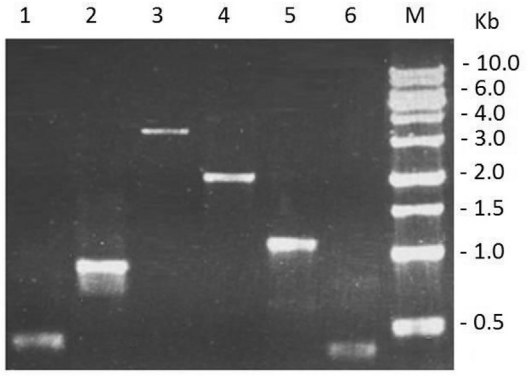

Fig. 1. Fragments amplified by PCR. Lane 1: GLSA (452 bp); Lane 2: GPD (817 bp); Lane 3: GLSB (3203 bp); Lane 4: GLSC (1708 bp); Lane 5: HYG (1043 bp); Lane 6: GLSD (335 bp); M: ladder 1 kb.

double and triple fusion, expected fragments should have 1317 bp (GLSA + GPD), 1430 bp (HYG + GLSD), 4501 bp (GLSA + GPD + GLSB) and 3095 bp (GLSC + HYG + GLSD), respectively, as shown by the arrows in Figs. 2 and 3. The presence of intermediaries suggests that the reaction is not totally completed, probably due to the presence of residual primers that re-amplify the same fragments. This effect is more evident in triple fusion, purification of the fragments being indicated when the objective is to assemble three or more fragments in the same reaction [15]. PCR products of the fused fragments showing 4501 bp (GLSA + GPD + GLSB) and 3095 bp (GLSC + HYG + GLSD) were mixed in equimolar quantities and fused in a new reaction. The

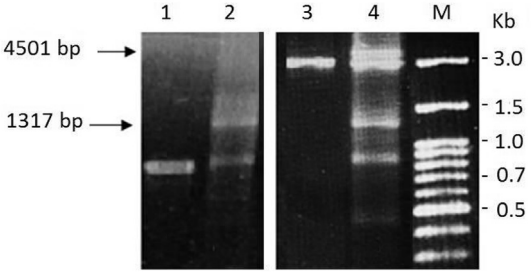

Fig. 2. PCR fusion-based double and triple fusion of fragments without purification. Lane 1: GPD (817 bp); Lane 2: GLSA + GPD (1317 bp); Lane 3: GLSB (3203 bp); Lane 4: GLSA + GPD + GLSB (4501 bp); M: ladder (100 bp).

Table 1
Composition of amplification primers and Tm (melting temperature).

Primer	Sequence (5′ to 3′)	Tm (°C)
GLSA-F[a]	gtaacgccagggttttcccagtcacgacgCAAAGGTTTCAAGCAAATGACATT	69.9
GLSA-R[b]	ATTCAGCGTCATCGAGAACTTCATACAAGACACTCTCCCGCCC	68.1
GPD-F[b]	GGGCGGGAGAGTGTCTTGTATGAAGTTCTCGATGACGCTGAATTCGTTG	68.7
GPD-R[b]	CTATCTCTTCTGCGGACATCACAAGGATGGGTGGTTGGGGA	68.6
GLSB-F[b]	TCCCCAACCACCCATCCTTGTGATGTCCGCAGAAGAGATAGAGGATA	69.3
GLSB-FD	ATYGATGCBAAYCARGACAAYTA	48.3
GLSB-RD[b]	CATYTGTTCDCCCATACCAG	50.8
GLSC-F[b]	CTGGTATGGGTGAACAGATGTTGAGTCGAGAGTACTACTATTTGGG	70.5
GLSC-R[b]	AGTTCAGGCATGGTGATGTCGATGCTTTTGCAGATAGAGCATT	67.0
HYG-F[b]	AATGCTCTATCTGCAAAAGCATCGACATCACCATGCCTGAACTCACCGC	69.3
HYG-R[b]	CGGGGGTCGCCAGATCTTTAGGTCGGCATCTACTCTATTCCTT	68.6
GLSD-F[b]	AAGGAATAGAGTAGATGCCGACCTAAAGATCTGGCGACCCCCGCAGTG	70.1
GLSD-R[a]	GCGGTTAACAATTTCTCTCTGGAAAcagcgctggggttatcgaaagtttcattg	67.9

[a] The lowercase letters correspond to the overlapping regions (29 bp) of the primers with the vector pRS426.
[b] The underlined portions correspond to the overlapping regions between the reverse (R) and the forward (F) primers of the subsequent fragments.