One-step enzymatic assembly of DNA molecules up to several hundred kilobases in size

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Method Article

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Abstract

Introduction

An isothermal, single-reaction protocol for assembling multiple, overlapping DNA molecules by the concerted action of a 5'-exonuclease, a DNA polymerase, and a DNA ligase is described. The DNA fragments are first recessed to produce ssDNA overhangs that are specifically annealed, and then they are covalently joined. This assembly protocol can be used to seamlessly construct synthetic and natural genes, genetic pathways, and entire genomes. This method could be a very useful molecular engineering tool.

Reagents

1. 5X isothermal \(ISO) reaction buffer \(25% PEG-8000, 500 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$, 50 mM DTT, 1 mM each of the 4 dNTPs, and 5 mM NAD). This is prepared as described below. 2. T5 exonuclease \(Epicentre) 3. Phusion DNA polymerase \(New England Biolabs) 4. Taq DNA ligase \(New England Biolabs)

Equipment

1. Heat block or thermocycler with PCR tubes.

Procedure

1. Prepare 5X ISO buffer. Six ml of this buffer can be prepared by combining the following: 3 ml of 1 M Tris-HCl pH 7.5 150 µl of 2 M $MgCl_2$ 60 µl of 100 mM dGTP 60 µl of 100 mM dATP 60 µl of 100 mM dTTP 60 µl of 100 mM dCTP 300 µl of 1 M DTT 1.5 g PEG-8000 300 µl of 100 mM NAD Add water to 6 ml Aliquot 100 µl and store at -20 °C 2. Prepare an assembly master mixture. This can be prepared by combining the following: 320 µl 5X ISO buffer 0.64 µl of 10 U/ µl T5 exo 20 µl of 2 U/µl Phusion pol 160 µl of 40 U/µl Taq lig Add water to 1.2 ml Aliquot 15 µl and store at -20 °C. This assembly mixture can be stored at -20 °C for at least one year. The enzymes remain active following at least 10 freeze-thaw cycles. This is ideal for the assembly of DNA molecules with 20-150 bp overlaps. For DNA molecules overlapping by larger than 150 bp, prepare the assembly mixture by using 3.2 µl of 10 U/ µl T5 exo. 3. Thaw a 15 µl assembly mixture aliquot and keep on ice until ready to be used. 4. Add 5 µl of DNA to be assembled to the master mixture. The DNA should be in equimolar amounts. Use 10-100 ng of each ~6 kb DNA fragment. For larger DNA segments, increasingly proportionate amounts of DNA should be added \(e.g. 250 ng of each 150 kb DNA segment). 5. Incubate at 50 °C for 15 to 60 min \(60 min is optimal). 6. If cloning is desired, electroporate 1 µl of the assembly reaction into 30 µl electrocompetent _E. coli_.

Timing

15-60 min