Manual

Fast-Link DNA Ligation Kits

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Fast-Link™ DNA Ligation Kits are part of the Epicentre™ product line, known for its unique genomics kits, enzymes, and reagents which offer high quality and reliable performance.

BIOSEARCH™ TECHNOLOGIES

GENOMIC ANALYSIS BY LGC

Manual

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Contents

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1. Introduction

The Fast-Link DNA Ligation Kits provide reagents optimised for rapid and efficient ligation of DNA. Ligation reactions require incubation for as little as 5 minutes at room temperature. Ligation reactions of PCR products containing nontemplate-dependent 3′ A-overhangs require incubation for just 1 hour at 16 °C. Applications include cloning DNA into prokaryotic or eukaryotic plasmid vectors and bacteriophage lambda vectors, and sample preparation for next-generation DNA sequencing.

2. Product designations and kit components

Product	Kit size	Catalog number	Reagent description	Part number	Volume
Fast-Link DNA Ligation Kit	50 ligations	LK0750H	Fast-Link DNA Ligase (2 U/µL)	E0077-2-D2	50 µL
			Fast-Link 10X Ligation Buffer	SS000272-D4	200 µL
			ATP (10 mM)	SS000391-D1	100 µL
	100 ligations	LK6201H	Fast-Link DNA Ligase (2 U/µL)	E0077-2-D3	100 µL
			Fast-Link 10X Ligation Buffer	SS000272-D5	400 µL
			ATP (10 mM)	SS000391-D3	200 µL

3. Product specifications

Storage: Store only at -20 °C in a freezer without a defrost cycle.

Storage buffer: Fast-Link DNA Ligase is supplied in a 50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 0.1% Triton® X-100 (Rohm & Haas) and 1 mM dithiothreitol.

Unit definition: One unit of Fast-Link DNA Ligase converts 1 nmol of pyrophosphate into Norit-adsorbable material in 20 minutes at 37 °C.[1]

Quality control: Fast-Link DNA Ligase activity is assayed in a reaction containing 33 mM Tris-acetate (pH 7.5), 66 mM potassium acetate, 10 mM magnesium acetate, 0.5 mM DTT, 1 mM ATP, 1 µg *Hin*d III-cut lambda DNA and 0.001-1.0 unit of enzyme.

Contaminating activity assays: Fast-Link DNA Ligase is free of detectable exonuclease, endonuclease and RNase activities as judged by agarose gel electrophoresis following incubation of 1 µg of DNA and RNA substrates with 150 U of the enzyme at 37 °C for 16 hours.

4. General considerations

1. **Preparation of vector and insert DNA molecules:** Prepare the vector and insert DNA molecules by restriction digestion, PCR amplification, or other physical/enzymatic methods. The ends of the DNA molecules must be compatible for ligation to occur efficiently. If necessary, separate the vector and insert from other contaminating molecules by any one of several methods, including electrophoretic, physical (e.g., columns), or organic extraction followed by ethanol precipitation. During purification of DNA molecules (e.g., using agarose gel electrophoresis), minimise exposure of insert and vector DNA to ultraviolet (UV) irradiation, which can cause formation of pyrimidine

dimers. Following purification, quantify the DNA by gel electrophoresis (comparing with a known amount of DNA) or fluorescence (e.g., using Hoechst dye 33258[2]).

2. **Dephosphorylation of vector DNA molecules:** Treatment of vector molecules to remove the 5′-phosphate groups will prevent recircularisation, minimising the recovery of nonrecombinant clones. This is especially important for vector molecules with compatible ends (nondirectional cloning).
3. **Cloning of PCR amplification products:** Phosphate groups must be added to either the 5′ end of primers used in the synthesis of PCR amplification products, or the amplification products themselves must be directly phosphorylated, if these products are cloned into a dephosphorylated vector. Alternatively, incorporate restriction endonuclease sites near the 5′ end of the PCR primers and, following amplification, digest the amplification products and the vector with the appropriate enzyme. If digestion at the restriction site is inefficient, the amplification products can be treated with a combination of: i) T4 Polynucleotide Kinase to add phosphate groups; ii) Klenow or T4 DNA Polymerase to blunt the ends; and iii) T4 DNA Ligase to form concatamers.[3] The concatamer is then digested with the restriction endonuclease and the linear fragment cloned into an appropriate vector.
4. **Ratio of vector DNA to insert DNA:**[4,5] In general, for ligation of fragments with cohesive ends, you can achieve the greatest number of recombinants when the concentration of insert DNA is 2X that of the concentration of vector DNA (i.e., a molar ratio of 2:1, insert:vector). A lower insert:vector ratio will favor recircularisation of the plasmid, whereas a higher ratio will favor formation of vectors containing multiple inserts. For a 3-kb vector, the optimum concentration of vector DNA is 10-20 ng/µL; for a 10-kb vector, the optimum concentration of vector DNA is 5-10 ng/µL. (A lower concentration of DNA is required for larger vectors, as the likelihood that the ends of the same molecule will interact is less than for smaller vectors.) To achieve a 2:1 molar ratio of insert:vector DNA, the concentration of insert DNA will depend upon its size relative to the size of the vector. Ligations of DNA molecules with blunt ends require higher concentrations of insert DNA to achieve the greatest number of recombinants.[6] We recommend a molar ratio of 5:1, insert:vector, for most blunt-end ligations. For ligations involving linkers, use a 100:1 molar ratio of linker to vector.[4]
5. **ATP concentration:** High concentrations of ATP (>0.5 mM) inhibit ligation of DNA molecules with blunt ends.[7]
6. **Enzymatic treatment of ligation reactions:** Following ligation, the DNA molecules may be treated in the Fast-Link Reaction Buffer with most common restriction endonucleases or nucleases highly specific for linear double-stranded DNA.
7. **Electroporation:** Aliquots (1-2 µL) of the ligation reaction may be used directly in electroporation without desalting.
8. **Time of ligation:** The ligation of DNA molecules with compatible cohesive ends occurs quickly at room temperature, being essentially complete within 5 minutes (Figure 1). The ligation of molecules with blunt ends proceeds more slowly, although a substantial amount of product is formed within 15 minutes (Figure 2). Note that the length of incubation for any of the ligation reactions may be extended overnight with no deleterious effects.

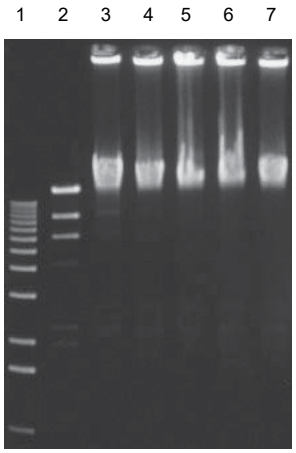

Figure 1. Time course for a cohesive-end ligation using the Fast-Link Kit. Lambda Hind III markers were ligated in a standard Fast-Link reaction using 2 U of Fast-Link DNA Ligase (lanes 3-7). Lane 1, 1-kb ladder; Lane 2, no enzyme; Lanes 3-7, 5 min, 15 min, 30 min, 1 hr, 2 hr, respectively.

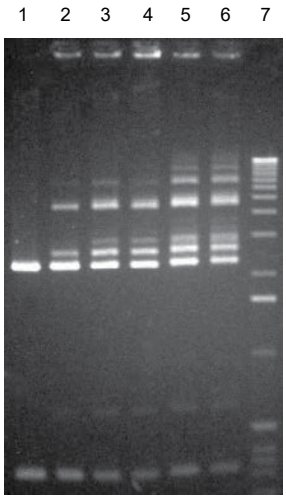

Figure 2. Time course for a blunt-end ligation using the Fast-Link Kit. pUC19 digested with Pvu II was ligated in a standard Fast-Link reaction using 2 U of Fast- Link DNA Ligase (lanes 2-6). Lane 1, no enzyme; Lanes 2-6, 5 min, 15 min, 30 min, 45 min, 1 hr, respectively; Lane 7, 1-kb ladder.

5. Kit procedure

5.A. Ligation of DNA molecules with cohesive or blunt ends

This protocol is suitable for the ligation of insert DNA with cohesive or blunt ends into plasmid vectors with compatible ends.

1. Assemble the reaction in a microcentrifuge tube at room temperature as outlined, adding the Fast-Link DNA Ligase last. (Numbers in parentheses indicate molar ratios.)

Ligations of insert DNA with cohesive ends

1.5	µL Fast-Link 10X Ligation Buffer
1.5	µL ATP (10 mM)
x	µL vector DNA (1)
y	µL insert DNA (2)
	sterile water to a volume of 14 µL
1	µL Fast-Link DNA Ligase
15	µL Total reaction volume

Ligations of insert DNA with blunt ends

1.5	µL Fast-Link 10X Ligation Buffer
0.75	µL ATP (10 mM)
x	µL vector DNA (1)
y	µL insert DNA (5)
	sterile water to a volume of 14 µL
1	µL Fast-Link DNA Ligase
15	µL Total reaction volume

2. Incubate the reaction at room temperature for 5 minutes (cohesive ends) or 15 minutes (blunt ends) (see Figures 1 and 2).
3. Transfer the reaction to a water bath or heat block at 70 °C for 15 minutes to inactivate the Fast-Link DNA Ligase; failure to inactivate the ligase may decrease transformation efficiencies.[6]
4. Centrifuge briefly in a microcentrifuge.
5. Transform competent *E. coli* cells with 1/10 of the ligation reaction, keeping the volume of the ligation to no more than 5% of the volume of competent cells,[4] or follow the manufacturer's recommendations. If electroporating the ligated molecules, use no more than 2 µL of the ligation reaction with 50 µL of electrocompetent cells.
6. To determine the extent of ligation, inactivate the ligase and analyse 5 µL of the ligation reaction by electrophoresis on an agarose gel.

5.B. Ligation of PCR products into T-vectors

This protocol is suitable for ligation of PCR products containing a nontemplate-dependent, 3′ A-overhang[8] into plasmids containing a 3′ T-overhang[9] (T-vectors). Separate PCR products from reaction components (e.g., template DNA and buffer salts). Prepare[10] or purchase a T-vector suitable for cloning PCR products.

1. Assemble the reaction in a microcentrifuge tube as outlined, adding the Fast-Link DNA Ligase last. (Numbers in parentheses indicate molar ratio.)

1.5 µL	Fast-Link 10X Ligation Buffer
0.75 µL	ATP (10 mM)
x µL	T-vector DNA (1)
y µL	PCR product (1)
	sterile water to a volume of 14 µL
1 µL	Fast-Link DNA Ligase
15 µL	Total reaction volume

2. Incubate the reaction at 16 °C for 1 hour.
3. Transfer the reaction to a water bath or heat block at 70 °C for 15 minutes to inactivate the Fast-Link DNA Ligase; failure to inactivate the ligase may decrease transformation efficiencies.[6]
4. Centrifuge briefly in a microcentrifuge.
5. Transform competent *E coli* cells with 1/10 of the ligation reaction, keeping the volume of the ligation to no more than 5% of the volume of competent cells,[4] or follow the manufacturer's recommendations. If electroporating the ligated molecules, use no more than 2 µL of the ligation reaction with 50 µL of electrocompetent cells.
6. To determine the extent of ligation, inactivate the and analyse 5 µL of the ligation reaction by electrophoresis on an agarose gel.

6. Troubleshooting

If few or no recombinant colonies are recovered:

1. **Check quantification of vector and insert DNA molecules.** Measure the concentration of DNA using electrophoresis or spectrophotometry and verify that the correct amounts of vector and insert are added to the reaction.
2. **Perform the ligation using a different ratio of vector to insert.** For cohesive-end ligations, try a molar ratio of 1:1 or 3:1 (insert:vector); for blunt-end ligations, try a molar ratio of 3:1 (insert:vector); for TA cloning of PCR products, try a molar ratio of 3:1 (insert:vector).
3. **Check that the ends of the DNA molecules are compatible.** Repeat enzymatic treatment of DNA molecules (e.g., restriction digestion; blunting or filling in using T4 DNA Polymerase or Klenow Fragment) to ensure that the ends of the vector and insert are compatible for ligation. For PCR products in which a restriction endonuclease site is part of the oligonucleotide, verify that the oligonucleotide has a sufficient number of bases 5′ of the restriction endonuclease recognition site. Repeat digestion using 20 U of the restriction endonuclease and digest overnight at room temperature (see Part 3).
4. **Check that the oligonucleotides used in PCR amplification have 5′-phosphate groups.** Repeat phosphorylation of oligonucleotides using fresh T4 Polynucleotide Kinase (see Part 3).
5. **Verify that competent cells are active.** Transform competent cells with 0.2-1.0 ng of circular plasmid and calculate transformation efficiency to ensure that competent cells are active.
6. **Verify the amount of DNA added to competent cells.** The addition of too much DNA (>5 ng total) will decrease the recovery of transformed colonies. If performing electroporation, addition of more than 2 µL of the ligation reaction may cause arcing.
7. **Verify the presence of 3′ A-overhangs on PCR products.** Use a DNA polymerase in PCR that does not possess a 3′”5′ exonuclease activity, which can remove 3′ A-overhangs. In addition, 3′ A-overhangs may be removed during agarose gel electrophoresis. 3′ A-overhangs may be added to PCR products by treatment with Taq DNA Polymerase and dATP.[11]
8. **Inactivate the ligase.** Nondenatured ligase may interact with the DNA, thereby preventing it from entering the bacterial cell.
9. **Verify use of the proper selective growth medium.**

If the number of nonrecombinant colonies is high:

1. **Remove 5′-phosphate groups from vector molecules.** Dephosphorylation of vector DNA molecules will prevent recircularisation. If cloning PCR products, dephosphorylation of the vector requires addition of phosphate groups to the 5′ end of the amplification products (see Part 3).
2. **Check linearisation of vector molecules.** Ensure that digestion of vector molecules is complete by using gel electrophoresis. If digesting with more than one restriction enzyme, treat first with the enzyme least likely to digest well and check for complete digestion before treatment with an additional enzyme.

7. References

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8. Further support

If you require any further support, please do not hesitate to contact our Technical Support Team: techsupport@lgcgroup.com.

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