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CircLigase™ II ssDNA Ligase

Cat. Nos. CL9021K and CL9025K

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1. Introduction

CircLigase™ II ssDNA Ligase† is a thermostable ligase that catalyzes intramolecular ligation (i.e., circularization) of single-stranded DNA (ssDNA) substrates that have both a 5′-monophosphate and a 3′-hydroxyl group. Linear ssDNAs of greater than ~15 bases are circularized by CircLigase II ssDNA Ligase. Under standard reaction conditions, virtually no linear concatamers or circular concatamers are produced.

2. Applications

Production of single-stranded DNA templates for rolling-circle replication or rolling-circle transcription experiments.

3. Kit Contents and Specifications

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

Storage Buffer: CircLigase II ssDNA Ligase is supplied in a 50% glycerol solution containing 50 mM Tris-HCl (pH 7.5), 100 mM NaCl, 0.1 mM EDTA, 1 mM dithiothreitol (DTT), and 0.1% Triton® X-100.

Unit Definition: One unit of CircLigase II ssDNA Ligase converts 1 pmol of a linear 5′-phosphorylated CircLigase II Control Oligo (55 mer) into exonuclease I-resistant circular ssDNA in 1 hour at 60°C under standard assay conditions.

CircLigase II 10X Reaction Buffer: 0.33 M Tris-acetate (pH 7.5), 0.66 M potassium acetate, and 5 mM DTT.

For circularization of ssDNA, we recommend adding $MnCl_2$ to a final concentration of 2.5 mM.

Contaminating Activity Assays: CircLigase II ssDNA Ligase is free of detectable DNA exonuclease and endonuclease, and RNase activities.

Component	Component Volumes	
	CL9021K (1,000 U)	CL9025K (5,000 U)
CircLigase™ II ssDNA Ligase (100 U/µl)	10 µl	50 µl
CircLigase™ II 10X Reaction Buffer	50 µl	150 µl
$MnCl_2$ (50 mM)	20 µl	75 µl
Betaine (5 M)	50 µl	250 µl
CircLigase™ ssDNA Control Oligo (2 pmol/µl)	10 µl	25 µl
Sterile Water	500 µl	1 ml

4. General Considerations

1. **Substrate Requirements:** The circularization reaction requires a ssDNA with 5′-phosphate and 3′-hydroxyl groups. The standard CircLigase II reaction uses 10 pmol of linear ssDNA.
2. **Substrate Size:** The ssDNA must be at least ~15 bases in length. Substrates such as single-stranded oligodeoxynucleotides and single-stranded cDNAs can be ligated by the enzyme.
3. **Manganese:** For circularization of ssDNA such as oligodeoxynucleotides or cDNA, add $MnCl_2$ to a final concentration of 2.5 mM. A tube of $MnCl_2$ is included
4. **Magnesium:** In general, circularization is better in the absence of magnesium.
5. **Amount of CircLigase II ssDNA Ligase in the Reaction:** The standard reaction conditions (Part 5) use 100 U of the CircLigase II enzyme per 20-µl reaction (~1 µM enzyme and 0.5 µM ssDNA substrate). For custom ligation reactions, we recommend maintaining the enzyme concentration in excess of the substrate concentration.
6. **Sequence Dependence:** Results at Epicentre indicate that the sequence of the ssDNA can influence the efficiency of the circularization reaction.
7. **Reaction Time:** The CircLigase II ssDNA circularization reaction is typically complete in 60 minutes. However, increasing the reaction time may improve the yield of circular DNA with difficult-to-ligate ssDNA substrates.
8. **Betaine:** Betaine is not necessary for circularization of easy-to-ligate ssDNA molecules. However, we have found that difficult-to-ligate ssDNA substrates can be circularized by including betaine at a final concentration of 1 M in the ligation reaction. A separate tube of betaine is provided in the kit to enable optimization of the betaine concentration, if necessary.
9. **Difficult Substrates:** Some ssDNAs are inefficiently circularized in the standard reaction (Part 5). The yield of circular ssDNA from a difficult-to-ligate substrate may be increased by increasing the concentration of CircLigase II ssDNA Ligase in the reaction, lengthening the reaction time (see Note 7, above), or by adding betaine to the reaction (see Note 8, above).
10. **The CircLigase II ssDNA Control Oligo:** The CircLigase II ssDNA Control Oligo provided in the kit is a 55-base oligodeoxynucleotide containing both 5′-phosphate and 3′-hydroxyl ends. Under standard reaction conditions (10 pmole Control Oligo, 100 U CircLigase II ssDNA Ligase, 2.5 mM $MnCl_2$, 1 hour reaction), the linear Control Oligo is converted to circular ssDNA.

5. Kit Procedure

5.A. Ligation Reaction

1. Combine the following reaction components:

			Final Concentration
x	µl	Sterile water	---
10	pmol	Single-stranded DNA	0.5 pmol/µl
2	µl	CircLigase II 10X Reaction Buffer	1X
1	µl	50 mM $MnCl_2$	2.5 mM
4	µl	5 M Betaine (optional)	1 M
1	µl	CircLigase II ssDNA Ligase (100 U)	5 U/µl
20	µl	Total reaction volume	

2. Incubate the reaction at 60°C for 1 hour.

   Note: *Longer incubation times may improve the yield of circular ssDNA for difficult-to-ligate ssDNAs. For example, we have observed that the ligation reaction with some ssDNAs went to completion in the presence of 1 M betaine after 16 hours of incubation.*

3. Incubate the reaction to 80°C for 10 minutes to inactivate the CircLigase II ssDNA Ligase.

5.B. Gel Analysis of the Ligation Reaction

The efficiency of a CircLigase II ligation reaction can be readily assessed by gel electrophoresis. When ligating oligos, load approximately 1 pmol of linear ssDNA substrate in one gel lane and 2 µl of the standard CircLigase II reaction mixture into an adjacent gel lane of a 20% acrylamide/8 M urea denaturing gel. Run the gel and stain with an appropriate DNA-binding dye. The circularized ssDNA product migrates slower, above, the linear ssDNA band (see Fig. 1). In some instances, the adenylated oligo-intermediate can be seen as a band just above the linear ssDNA.

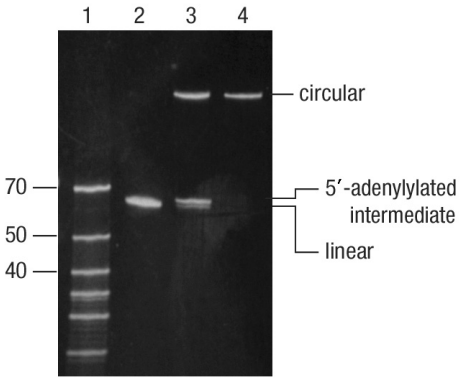

Figure 1. CircLigase™ II ssDNA Ligase converts linear ssDNA into closed circular ssDNA. A 71-nucleotide ssDNA oligo was converted to a circular ssDNA. Lane 1, DNA markers; lane 2, 71-nucleotide linear ssDNA oligo; lane 3, circularization proceeds through an adenylated intermediate; lane 4, closed-circular ssDNA reaction product.

5.C. Removing the Linear ssDNA Substrate and Adenylated Intermediate from the Reaction

Once the CircLigase II reaction has been terminated, the remaining linear ssDNA substrate and linear single-stranded adenylated intermediate can be removed by treatment with Exonuclease I (which digests linear ssDNA) and Exonuclease III (which digests linear double-stranded DNA). The circular ssDNA is resistant to these exonucleases, while the linear ssDNA and adenylated intermediate are digested.

Most linear ssDNA and adenylated-intermediate can be eliminated by addition of 20 U of Exonuclease I, followed by incubation at 37°C for 45 minutes.

However, if the linear ssDNA substrate contains hairpins or other secondary structures, treatment with both Exonuclease I and Exonuclease III is recommended. We suggest incubating a standard ligation reaction mixture with 10 U of Exonuclease I and 100 U of Exonuclease III at 37°C for 45 minutes.

6. Related Products

Cat. #	Concentration	Quantity
CircLigase™ II ssDNA Ligase		
CL9021K		1,000 Units
CL9025K		5,000 Units
Includes: CircLigase™ II ssDNA Ligase, CircLigase™ II 10X Reaction Buffer, 50 mM $MnCl_2$, CircLigase™ ssDNA Control Oligo, Betaine, Sterile Water.		
Exonuclease I, *E. coli*		
X40501K	20 U/µl	1,000 Units
X40505K	20 U/µl	5,000 Units
X40520K	20 U/µl	20,000 Units
Exonuclease III, *E. coli*		
EX4405K	200 U/µl	5,000 Units
EX4425K	200 U/µl	25,000 Units
Includes 10X Reaction Buffer.		

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