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CircLigase™ ssDNA Ligase

Cat. Nos. CL4111K and CL4115K

1. Introduction

CircLigase™ ssDNA Ligase† is a thermostable ATP-dependent ligase that catalyzes intramolecular ligation (i.e., circularization) of single-stranded DNA (ssDNA) and single-stranded RNA (ssRNA) substrates that have both a 5′-monophosphate and a 3′-hydroxyl group. Linear ssDNAs and ssRNAs of greater than ~30 bases are circularized by CircLigase ssDNA Ligase. Under standard reaction conditions, virtually no linear concatamers or circular concatamers are produced.

2. Product Designations and Kit Components

Product	Kit Size	Catalog Number	Reagent Description	Part Numbers	Volume
CircLigase™ ssDNA Ligase	1,000 Units	CL4111K	CircLigase™ ssDNA Ligase (100 U/µL)	E0129-100D5	10 µL
			$MnCl_2$ (50 mM)	SS000578-D2	20 µL
			ATP (1 mM)	SS000579-D1	20 µL
			CircLigase™ 10X Reaction Buffer	SS000581-D1	50 µL
			CircLigase™ ssDNA Control (2 pmole/µL)	SS000592-D1	10 µL
			Nuclease-Free Water, Sterile	SS000772-D3	1 mL
	5,000 Units	CL4115K	CircLigase™ ssDNA Ligase (100 U/µL)	E0129-100D2	50 µL
			$MnCl_2$ (50 mM)	SS000578-D3	75 µL
			ATP (1 mM)	SS000579-D2	75 µL
			CircLigase™ 10X Reaction Buffer	SS000581-D2	150 µL
			CircLigase™ ssDNA Control (2 pmole/µL)	SS000592-D2	25 µL
			Nuclease-Free Water, Sterile	SS000772-D3	1 mL

3. Product Specifications

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

Storage Buffer: CircLigase 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 ssDNA Ligase converts 1 pmol of a linear 5′-monophosphorylated CircLigase Control Oligo (55-mer) into an exonuclease I-resistant circular form in 1 hour at 60°C under standard assay conditions.

CircLigase 10X Reaction Buffer: 0.5 M MOPS (pH 7.5), 0.1 M KCl, 50 mM $MgCl_2$, and 10 mM DTT.

ATP is added to the reaction to a final concentration of 0.05 mM ATP. For additional optimization, $MnCl_2$ can be added to a final concentration of 2.5 mM $MnCl_2$ (see Note 3, Part 4).

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

4. Applications

- Production of single-stranded DNA templates for rolling-circle replication or rolling-circle transcription experiments and next-generation sequencing.
- Production of circular ssRNA >30 nt.

5. General Considerations

1. **Substrate Requirements:** The circularization reaction requires a ssDNA or ssRNA with 5′-phosphate and 3′-hydroxyl groups. The standard CircLigase reaction uses 10 pmol of linear ssDNA.
2. **Substrate Size:** The ssDNA or ssRNA 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. **$MnCl_2$:** Generally, circularization of ssDNA or ssRNA, such as oligodeoxynucleotides or cDNA, is enhanced by the addition of manganese chloride ($MnCl_2$) to the reaction to a final reaction concentration of 2.5 mM. A tube of $MnCl_2$ is included.
4. **Amount of CircLigase ssDNA Ligase in the Reaction:** The standard reaction conditions (Part 5) use 100 U of the CircLigase 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.
5. **Sequence Dependence:** Our results indicate that the sequence of the ssDNA can strongly influence the efficiency of the circularization reaction.
6. **Reaction Time:** The CircLigase 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.
7. **Difficult Substrates:** Some ssDNAs or ssRNAs 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 ssDNA Ligase in the reaction or lengthening the reaction time (see Note 6, above).
8. **Control Template:** The CircLigase 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 pmol Control Oligo, 100 U CircLigase ssDNA Ligase, 2.5 mM $MnCl_2$, 1-hour reaction), the linear Control Oligo is converted to circular ssDNA.

6. Kit Procedure

6.A. Ligation Reaction

1. Combine the following reaction components:

			Final Concentration
x	µL	Sterile water	---
10	pmol	Single-stranded DNA or RNA template	0.5 pmol/µL
2	µL	CircLigase 10X Reaction Buffer	1X
1	µL	1 mM ATP	50 µM
1	µL	50 mM $MnCl_2$	2.5 mM
1	µL	CircLigase 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 or larger amounts of CircLigase ssDNA Ligase may improve the yield of circular ssDNA.*

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

6.B. Gel Analysis of the Ligation Reaction

The efficiency of a CircLigase 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 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.

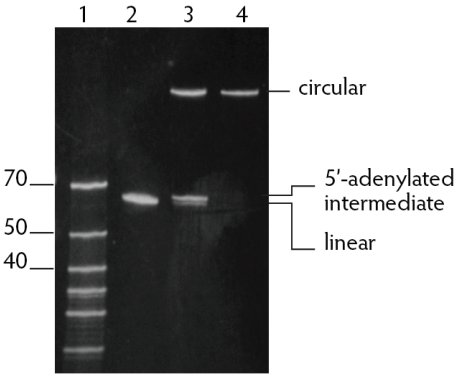

Figure 1. CircLigase™ ssDNA Ligase converts linear ssDNA into closed circular ssDNA. A 71-nucleotide ssDNA oligo was converted to a circular ssDNA. Lane 1, DNA markers; lane 2, 71-nucleotide linear ssDNA oligo; lane 3, circularization proceeds through an adenylated intermediate; lane 4, closed-circular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

6.C. Removing the Linear ssDNA Template and Adenylated Intermediate from the Reaction

Once the CircLigase 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. Single-stranded linear nucleic acids that were not circularized in the CircLigase reaction can be removed by digestion with Exonuclease I (for DNA), or Terminator™ Exonuclease or RNase R (for RNA).

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 structure, treatment with both Exonuclease I and Exonuclease III may be required. 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.

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