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# CircLigase™ II ssDNA Ligase

Cat. Nos. CL9021K and CL9025K

## 1. Introduction

CircLigase™ II ssDNA Ligase† is a thermostable 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™ II ssDNA Ligase | 1,000 Units | CL9021K | CircLigase™ II ssDNA Ligase (100 U/µL) | E0129-100D3 | 10 µL |
| | | | Betaine (5M) | SS000026-D4 | 50 µL |
| | | | $MnCl_2$ (50 mM) | SS000578-D2 | 20 µL |
| | | | CircLigase™ ssDNA Control (2 pmole/µL) | SS000592-D1 | 10 µL |
| | | | Nuclease-Free Water | SS000772-D3 | 1 mL |
| | | | CircLigase™ II 10X Reaction Buffer | SS000881-D1 | 50 µL |
| | 5,000 Units | CL9025K | CircLigase™ II ssDNA Ligase (100 U/µL) | E0129-100D4 | 50 µL |
| | | | Betaine (5M) | SS000026-D5 | 250 µL |
| | | | $MnCl_2$ (50 mM) | SS000578-D3 | 75 µL |
| | | | CircLigase™ ssDNA Control (2 pmole/µL) | SS000592-D2 | 25 µL |
| | | | Nuclease-Free Water | SS000772-D3 | 1 mL |
| | | | CircLigase™ II 10X Reaction Buffer | SS000881-D2 | 150 µL |

## 3. Product 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.

## 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 II 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. **Manganese:** For circularization of ssDNA or ssRNA, 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:** Our results 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. In some cases, the molar concentration of the substrate DNA may be high enough that ligation may not be complete; it may be helpful to add 1 µL of 1 mM ATP to allow the ligation reaction to proceed to completion.
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 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 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.

## 6. Kit Procedure

### 6.A. Ligation Reaction

1. Combine the following reaction components:

| | | Final Concentration |
|---|---|---|
| x µL | Nuclease-Free Water | --- |
| 10 pmol | Single-stranded DNA or RNA template | 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.

### 6.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.

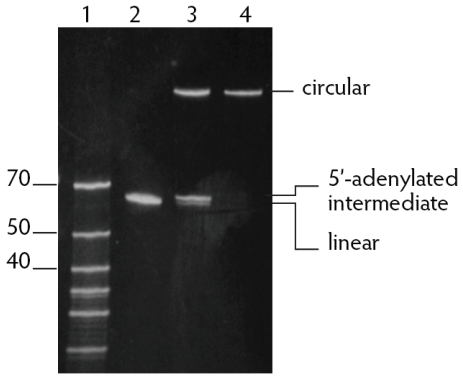


**Figure 1. CircLigase™ II ssDNA Ligase converts linear ssDNA into closed circular ssDNA.** A 71-nucleotide ssDNA oligo was converted to a circular ssDNA. Lane 1, DNA markers; lane 2, 71-nucleotide linear ssDNA oligo; lane 3, circularization proceeds through an adenylated intermediate; lane 4, closed-circular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

### 6.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. 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 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.