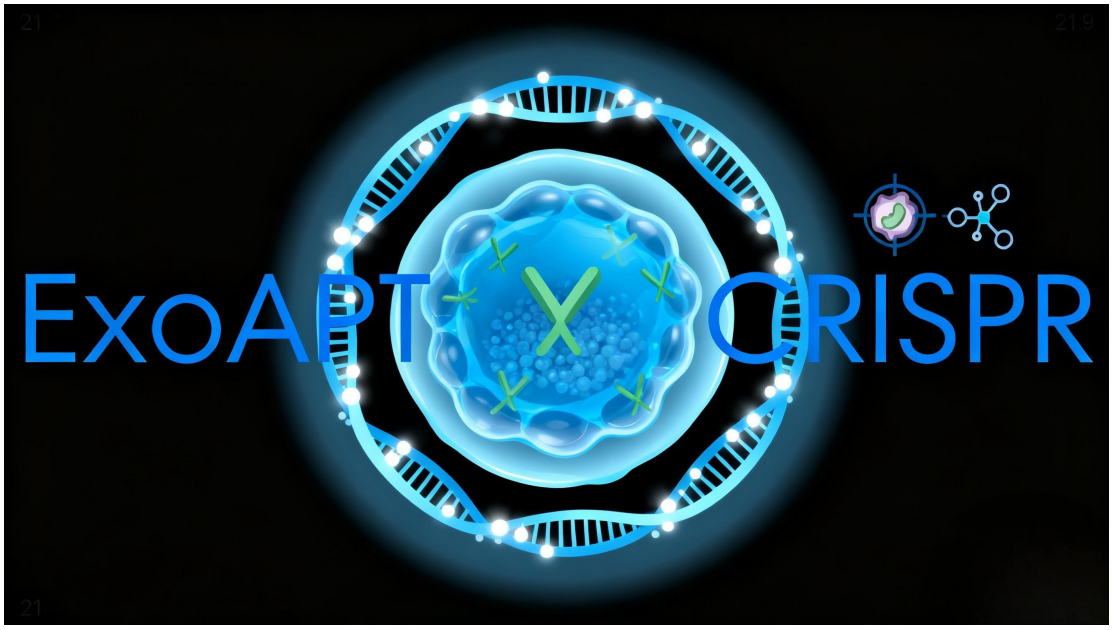


RCA Template

Wet Lab

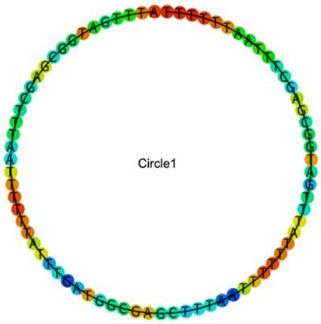
ZZU-China, October 2025

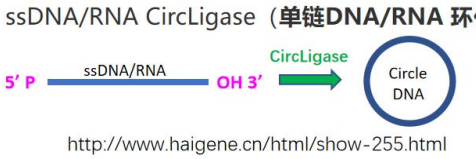
Contents

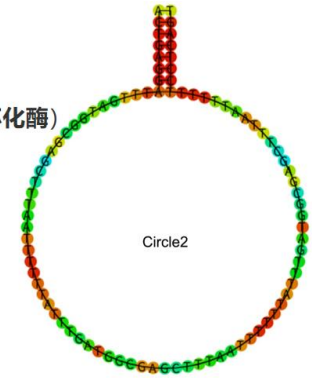
RCA Template

1. Design

Since Cas14a uses sgRNA (equivalent to crRNA in Cas12a), the sequence is as follows:
CUUCACUGAUAAAGUGGAGAACCGCUUCACCAAAAGCUGUCCCUUAGGGGAU
UAGAACUUGAGUGAAGGUGGGCUGCUUGCAUCAGCCUAAUGUCGAGAAGUG
CUUUCUUCGGAAAGUAACCCUCGAAACAAAUUCAUUUUUCCUCUCCAAUUCU
GCACAAGAAAGUUGCAGAACCCGAAUAGACGAAUGAAGGAAUGCAAC + guide sequence
guide sequence=AUUUGAUGGCGAGCUUUAAU
sgRNA**sequence**：
CUUCACUGAUAAAGUGGAGAACCGCUUCACCAAAAGCUGUCCCUUAGGGGAU
UAGAACUUGAGUGAAGGUGGGCUGCUUGCAUCAGCCUAAUGUCGAGAAGUG
CUUUCUUCGGAAAGUAACCCUCGAAACAAAUUCAUUUUUCCUCUCCAAUUCU
GCACAAGAAAGUUGCAGAACCCGAAUAGACGAAUGAAGGAAUGCAACAUUUGA
UGGCGAGCUUUAAU
The circular ssDNA is designed using the **Complement Target sequence** as the main unit, repeated three times and connected end-to-end, forming the **circle1** sequence. To improve ligation efficiency, reverse complementary sequences **ACTGAGG** (at 5') and **CCTCAGT** (at 3') are added to both ends (forming **circle2**), so that after annealing, the 5' and 3' ends are brought into close proximity, facilitating circularization by CircLigase.







2.Preparation of Circular DNA

2.1Materials

- circle2 sequence (5'-phosphorylated, HPLC purified)
- CircLigase
- 2.5× CircLigase Buffer
- 50 mM $MnCl_2$

- Exonuclease I
- 10× EXO1 Buffer
- DNA Purification Kit
- Ultrapure Water
- Constant Temperature Water Bath
- Nanodrop Spectrophotometer

2.2Circularization Reaction

1. Synthesize the **circle2** sequence with 5'-phosphorylation modification and HPLC purification.
2. Denature the **circle2** sequence at 94°C for 3 min, then cool to room temperature before use.

Table 1. Circularization Reaction System Setup

Reagent	Volume
CircLigase(100 U/μL)	2μL
Circle2sequence（10μM）	20μL
2.5×CircLigase Buffer	16μL
$MnCl_2$(50mM)	2μL

3. **Circularization reaction:** Incubate at 60°C for 60 min. After completion, heat-inactivate CircLigase at 85°C for 10 min to prevent interference in downstream steps.

2.3 Removal of Residual Linear ssDNA

Use Exonuclease I to degrade any unligated linear ssDNA remaining after the circularization reaction.

Table 2. Degradation Reaction System Setup

Reagent	Volume
Exonuclease I Buffer	6μL
Exonuclease I	6μL
Circularization product	40μL
ddH_2O	Up to 60μL

Incubate at 37°C for 30 minutes. Then, inactivate Exonuclease I by heating at 80°C for 15 min.

2.4 Purification

Use a small-scale DNA purification kit according to the manufacturer's instructions to purify the product, eluting in 50 μL to obtain circular ssDNA.

2.5 Measure cssDNA Concentration

Determine concentration using Nanodrop at 260/280 nm

3.Rolling Circle Amplification (RCA)

3.1Materials

- 10× Phi29 Buffer
- dNTP Mix
- Phi29 DNA Polymerase
- Target strand
- Circular DNA prepared in previous step
- DEPC-treated Water
- CRISPR Reagents

3.2Optimization of Circular DNA Concentration

Take 10 μL each of circular DNA at concentrations of 100 nmol/L, 500 nmol/L, and 800 nmol/L, respectively. Mix each with 4 μL of target strand (5 nmol/L). Perform duplex annealing at 94°C for 5 min. After annealing, slowly cool the reaction system to room temperature to ensure stable double-stranded formation. Then add to the phi29 reaction system for amplification.

Table 3. Reaction System Setup

Component	**Volume**(μL)
10× Phi29 Buffer	2
dNTP Mix (10 mM)	2
Phi29 DNA Polymerase	1
Annealing products	14
DEPC-treated water	1

Then, take 20 μL of the RCA product and add it to the CRISPR reaction system (total volume 40 μL).