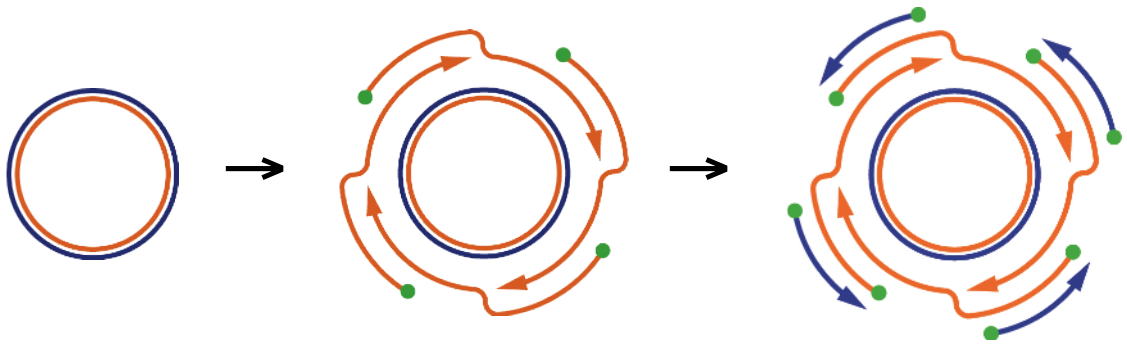
INTRODUCTION

Rolling Circle Amplification (RCA)[1-3] is a powerful isothermal nucleic acid amplification technique particularly useful for amplifying circular DNA constructs, such as plasmids. The process begins with a DNA template and short DNA primers. Phi29 DNA Polymerase extends the primers, continuing to synthesize DNA around the circular template by displacing the previously synthesized DNA strand when encountered (see Fig. 1). Unlike Polymerase Chain Reaction, RCA is an isothermal reaction, eliminating the need for thermal cycling and allowing for ease of reaction scalability. Key features of Phi29 DNA polymerase include: 1) high-processivity, incorporating tens of thousands of nucleotides without dissociating from the template, 2) strand displacement ability to dislodge the previously synthesized DNA strand allowing for continuous amplification of the template and 3) a very low error rate (~1 in 10^6-10^7 bases) resulting from inherent 3'→5' proofreading exonuclease activity, ensuring accurate amplification.[4,5]

Figure 1



The advantages of RCA are sensitivity, specificity and simplicity:

a) RCA can amplify from very small amounts of starting material as low as 5 pg.

b) The preference for circular templates reduces non-specific amplification.

c) Requires minimal equipment.

The protocol provided with the TempliAMP™ RCA Kit is versatile and can be applied to various types of circular DNA templates:

a) Amplification of purified DNA.

b) Direct amplification from bacterial sources containing circular DNA template of interest, such as plasmid. This protocol eliminates the need for DNA extraction, allowing for RCA of circular DNA template directly from colonies on agar plate, glycerol stocks and liquid media cultures.

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MATERIALS

Materials Supplied

Important Store at –20°C in a freezer without a defrost cycle. Do not store at –70°C.

TempliAMP™ RCA Kit Contents (25 reactions)	
Kit Component	Reagent Volume
TempliAMP™ Phi29 Enzyme Solution in 50% glycerol, 50 mM Tris-HCl, pH 7.5, 100 mM NaCl, 1 mM dithiothreitol (DTT), 0.1 mM EDTA and 0.1% Triton® X-100.	65 µl
10X TempliAMP™ Reaction Buffer	125 µl
25 mM dNTP Solution	50 µl
500 µM Random Primer Mix	65 µl
100 mM Dithiothreitol (DTT)	50 µl
RNase-Free Water	3 x 1.6 ml

Random Primer Mix

5'- **N N N N N*N*N** -3' where N = any DNA base and

* = Phosphorothioate bond (to prevent degradation by TempliAMP Phi29 proofreading activity)

Materials Required, but not Supplied

- AMPure® XP Bead-based Reagent (Beckman Coulter) for DNA clean-up
  See the Technical Appendix for alternative purification options.
- Thermocycler or heat block capable of 95°C incubation.
- 70% ethanol
- Optional: Materials for agarose gel electrophoresis and visualization
- Optional: Materials for fluorescence-based DNA quantification

SPECIFICATIONS

Functional Testing

The TempliAMP RCA Kit is functionally tested to ensure ≥30 µg yields of DNA from a 50 µl reaction using 5 ng of plasmid as starting template set up following the protocol presented in this TempliAMP RCA Kit literature.

Contaminating Activity Assays

All components of the TempliAMP RCA Kit are free of detectable RNase and DNase activities except for the inherent proof-reading activity of the TempliAMP Phi29 DNA Polymerase.

TempliAMP Phi29 Enzyme *E. coli* genomic DNA content measures below the lowest level of detection (<10 copies per 100 ng) as assayed by qPCR.

For more information, consult the appropriate safety data sheet (SDS) at www.cellscript.com.

PROCEDURE

A. TempliAMP RCA Reaction

1. In a sterile tube combine the following reagents at room temperature in the order indicated below:

TempliAMP RCA Reaction (step 1) (Template denaturation and primer annealing)	
Component	Amount
RNase-Free Water	35 µl
10X TempliAMP Reaction Buffer	5 µl
500 µM Random Primer Mix	2.5 µl
25 mM dNTPs	2 µl
Template DNA (5 pg–100 ng)	1 µl
Total Volume	45.5 µl

2. Mix gently and briefly centrifuge to collect the reagents in the bottom of the tube.

3. Incubate at 95°C for 3 minutes in a thermocycler or heat block.

4. Place the tube at ambient temperature and allow to cool to room temperature (approximately 3-4 minutes).

5. Once cooled, add the following components to the reaction:

TempliAMP RCA Reaction (step 2) (Rolling Circle Amplification)	
Component	Amount
Denatured/annealed Mixture (from step 1)	45.5 µl
100 mM DTT	2 µl
TempliAMP Phi29 Enzyme Solution	2.5 µl
Total Reaction Volume	50 µl

6. Gently mix the reaction by pipetting up and down several times.

7. Incubate the reaction at 30°C for 4-5 hours in a thermocycler or heat block. If a thermocycler is used, lid temperature should be set for ≤40°C to avoid inadvertent heat inactivation of the TempliAMP Phi29 Enzyme Solution. Lower DNA inputs can benefit from the full 5 hour incubation time to maximize yield.

8. Incubate the reaction at 65°C for 10 minutes to stop the reaction by heat inactivation. This step is critical to perform prior to restriction digest due to the inherent 3'→5' proofreading exonuclease activity of Phi29 DNA Polymerase. After heat inactivation the RCA product can now to be used as is (store overnight at 4°C or –20°C for longer term storage) or purified (see Step B: Purification and Quantification of the RCA Reaction Product). If the RCA product is too vicious it can be diluted with RNase-Free water.

B. Purification and Quantification of the RCA Reaction Product

Unincorporated dNTPs will be present in the unpurified RCA reaction so if it is desired to proceed without purification, quantify yield by fluorometric methods (e.g., Quant-it™ PicoGreen™ or Qubit® Fluorometer [both from Thermo Fisher Scientific]), then use directly in downstream applications. Alternatively, the RCA reaction product can be purified by standard DNA purification methods. Choose a method which will remove the unincorporated dNTPs from the mixture thus permitting quantification via A_{260} absorbance. We recommend AMPure XP Bead-based Reagent (Beckman Coulter) for this purpose since this method will remove all unincorporated dNTPs as well as avoid mechanical shearing of the very large RCA product which could happen if using a precipitation-based purification method. A protocol for AMPure XP Bead purification is given below. For alternative DNA purification methods, see the Technical Appendix.

1. Allow beads to warm to room temperature.

2. Vortex the beads prior to use. Add 90 µl of AMPure XP beads to the completed 50 µl RCA reaction.

3. Pipet the samples up and down several times until thoroughly mixed.
   Let sit at room temperature for 5-10 minutes with occasional mixing by finger flicking the side of the tube. If there is a lot of RCA product present, the beads will start to clump together. This is normal.

4. After the incubation, place the tube on a magnetic plate for 5-10 minutes or until the solution is clear. Without disturbing the pellet, carefully aspirate the solution off the samples and discard it.

5. With samples still magnetized, dispense 180 µl of 70% ethanol (freshly prepared) into the tube. Incubate at room temperature for 30-60 seconds. Carefully aspirate and discard the supernatant.

6. Remove from the magnet. Air-dry the bead pellets at room temperature for 4-10 minutes or until cracks appear at the edges of the beads.

7. Add 100-150 µl of RNase-Free Water to the tube and mix by finger flicking the side of the tube. The more RCA product present in the reaction, the longer the required resuspension time will be for the DNA to go back into solution. This process can take 5–30 minutes. Finger flick the tube every 5-10 minutes to aid the resuspension process. Ensure that all of the beads are homogeneously dispersed in solution (indicating DNA resuspension) before proceeding to the next step.

8. Place the tube on a magnetic plate for 5-10 minutes or until the solution is clear. Once the solution is clear, remove the supernatant and transfer to a new tube. This is your purified RCA reaction product.

9. The RCA products can be verified by restriction digest or DNA sequencing.

10. Purified RCA reaction products can be quantified by A_{260} absorbance and used for downstream applications.

C. Amplification of Plasmid DNA Directly from Bacterial Cells

Plasmid-containing bacterial cells can be used as an RCA template source directly from agar plates, liquid cultures or glycerol stocks.

a. **For bacterial colonies:**
   - Dispense 9 µl of RNase-Free Water into a microfuge tube.
   - Pick a single colony from an agar plate using a micropipet tip and resuspend the cells in the water by pipetting up and down 10 times or until fully resuspended.

b. **For bacterial liquid cultures or glycerol stock cultures:**
   - Dispense 9 µl of RNase-Free Water into a microfuge tube.
   - Add 1 µl of bacterial liquid culture and briefly mix to resuspend.
     If RCA reaction yield is lower than desired, increase the amount of culture used up to 10 µl.

1. In a sterile tube, combine the following reagents at room temperature in the order indicated below:

TempliAMP RCA Reaction (step 1) (Template denaturation and primer annealing)	
Component	Amount
RNase-Free Water	x µl
10X TempliAMP Reaction Buffer	5 µl
500 µM Random Primer Mix	2.5 µl
25 mM dNTPs	2 µl
Bacterial Resuspension	1-10 µl
Total Volume	45.5 µl

2. Mix gently and briefly centrifuge to collect the reagents in the bottom of the tube.

3. Mix gently and incubate at 95°C for 3 minutes in a thermocycler or heat block.

4. Place the tube at ambient temperature and allow to cool to room temperature (approximately 3-4 minutes).

5. Once cooled, add the following components to the reaction:

TempliAMP RCA Reaction (step 2) (Rolling Circle Amplification)	
Component	Amount
Denatured/annealed Mixture (from step 1)	45.5 µl
100 mM DTT	2 µl
TempliAMP Phi29 Enzyme Solution	2.5 µl
Total Reaction Volume	50 µl

6. Gently mix the reaction by pipetting up and down several times.

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7. Incubate the reaction at 30°C for 4-5 hours in a thermocycler or heat block. If a thermocycler is used, lid temperature should be set for ≤40°C to avoid inadvertent heat inactivation of the TempliAMP Phi29 Enzyme Solution. Lower DNA inputs can benefit from the full 5 hour incubation time to maximize yield.

8. Incubate the reaction at 65°C for 10 minutes to stop the reaction by heat inactivation. This step is critical to perform prior to restriction digest due to the inherent 3'→5' proofreading exonuclease activity of Phi29 DNA Polymerase. After heat inactivation the RCA product can now to be used as is (store overnight at 4°C or –20°C for longer term storage) or purified (see Step B: Purification and Quantification of the RCA Reaction Product). If the RCA product is too vicious it can be diluted with RNase-Free water.

TROUBLESHOOTING

Symptom	Solution
Low or No rolling circle amplification product observed	Too little starting DNA template was used in the reaction. • Increase amount of input DNA template ≥5 pg.
	DTT reagent has become oxidized making it inactive. • Repeated freeze-thaw cycles can reduce the activity of DTT. Repeat the RCA reaction with fresh DTT.
	If AMPure XP beads were used for purification, RCA reaction product was not fully solubilized before proceeding. • Repeat the RCA reaction making sure that all of the beads can be homogeneously dispersed in the solution before proceeding. If the beads are still clumpy after 30 minutes, continue incubation and mix the tube every 15 minutes until the beads can be homogeneously dispersed.
	Inhibitors are present in the input DNA sample. • Purify the DNA prior to use in RCA to remove the inhibitors.
	Random primers lacking phosphorothioate bonds on the 3' end were used. Phi29 DNA polymerase digested the primers. • Use the Random Primer Mix provided in the kit.
RCA reaction product is resistant to restriction enzyme digestion	High levels of genomic DNA (gDNA) are present in the input DNA prep. • If using low copy-number plasmid DNA as input DNA, treat the plasmid prep with RecBCD nuclease (Exo V) to degrade the gDNA and repeat the RCA reaction.

TECHNICAL APPENDIX

RCA Reaction Product Purification

Purify the RCA reaction product DNA by your preferred method. The method chosen should remove residual proteins and unincorporated dNTPs from the DNA. Several options are listed below.

I) **DNA-Binding Purification Column**: Several options are available commercially from multiple vendors. Follow the manufacturer's recommended protocol.

II) **Salt / Ethanol Precipitation**: We **do not** recommend using a precipitation-based method of cleanup to avoid mechanical shearing of the very large RCA product DNA.

REFERENCES

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