phi29-XT RCA Kit

NEB #E1603S/L

100/500 reactions

Version 1.0_3/23

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Kit Components and Storage Conditions

The kit should be stored at -20°C upon receipt and has a shelf life of 24 months when stored properly. The phi29-XT Reaction Buffer, Exonuclease-Resistant Random Primers and Deoxynucleotide (dNTP) Solution Mix remain frozen at -20°C and are stable for at least 30 freeze/thaw cycles. Thaw frozen components at room temperature, and then place all components on ice or at 4°C during use. Store materials at -20°C after use.

- phi29-XT DNA Polymerase, 10X
- phi29-XT Reaction Buffer, 5X
- Exonuclease-Resistant Random Primers, 500 µM
- Deoxynucleotide (dNTP) Solution Mix, 10 mM

Required Equipment/Materials Not Included

- Circular DNA template (either single- or double-stranded)
- Nuclease-free water
- Thin-walled, nuclease-free PCR tubes/plates or microcentrifuge tubes
- Thermocycler

Introduction

Rolling Circle Amplification (RCA) is a robust and highly sensitive isothermal amplification approach to continuously amplify circular DNA, generating long, repetitive copies of the circular sequence.

This kit features an engineered phi29 DNA Polymerase, phi29-XT. phi29-XT DNA Polymerase generates more DNA product in a shorter amount of time than wild-type phi29 DNA Polymerase, while sharing key qualities that are ideal for RCA applications including high processivity, strong strand displacement activity, and high-fidelity. phi29-XT is also more thermostable, with an optimal reaction temperature of 42°C, and has improved sensitivity over wild-type, supporting amplification down to 1 fg of DNA input. This kit also includes dNTPs and exonuclease-resistant random primers to universally amplify circular DNA sequences.

General Tips and Considerations

Input material

- This kit is compatible with 1) purified circular DNA (single- or double-stranded), or 2) direct amplification from bacteria containing circular template. A specific protocol is provided in Section II for amplification of bacterial DNA from agar plate colonies, liquid media cultures, and glycerol stocks without the need for DNA extraction.

Primers

- RCA with random primers allows amplification of circular DNA sequences without needing site-specific primers. RCA performed with random primers will generate branched double-stranded DNA products.
- phi29-XT DNA Polymerase, like wild-type phi29 DNA Polymerase, has strong 3´→5´ exonuclease activity for proofreading. The random primers provided in this kit are exonuclease-resistant by the addition of phosphorothioate bonds to protect them from degradation by phi29-XT. When using target-specific primers rather than the random primers provided in this kit, it is recommended that the primers are protected with a minimum of two phosphorothioate bonds on the 3´ end.

Reaction Temperature

- While wild-type phi29 DNA Polymerase reactions are typically carried out at 30-37°C, phi29-XT DNA Polymerase is more thermostable and works optimally at 42°C. Amplification below 37°C using this kit is not recommended.

Incubation time

- RCA reactions with phi29-XT DNA Polymerase should be performed at 42°C for 2 hours. For extremely low input amounts (e.g., 1 fg of 2 kb plasmid), longer incubation times may increase product yield.
- In the absence of template, non-specific DNA products may be produced when RCA reactions are incubated beyond 2 hours. However, when a circular template is present, incubation over 2 hours does not typically increase background amplification.

RCA Products

- RCA with random primers generates long, branched double-stranded DNA products. These products are suitable for many downstream applications without any further processing steps.
- For applications where the branched nature of the RCA products may be disruptive (e.g., nanopore-based DNA sequencing), debranching using T7 Endonuclease I (NEB #M0302) may be helpful. See the debranching protocol for details.
- A typical RCA reaction with phi29-XT DNA Polymerase will yield >5 µg of DNA from 1 pg of 2 kb circular DNA input in 2 hours. Increasing the initial circular template concentration or incubation time may increase the product yield.

RCA Product Quantitation and Verification

- RCA products may be directly quantified after dilution by Quant-iT® PicoGreen® dsDNA Assay Kit or Qubit® Fluorometer.
- Purified RCA products can be quantitated by measuring the absorbance at 260 nm or by NanoDrop®.
- RCA product quality can be verified by digesting with a restriction enzyme and running the products on an agarose gel.

Protocols for amplification of DNA using the phi29-XT RCA Kit

Section I. phi29-XT RCA with purified circular DNA input

Input Material: Purified single- or double-strand circular DNA

1. Prepare reactions without enzyme as described in the table below. Mix thoroughly, but gently, by pipetting or vortexing. Centrifuge briefly to collect solutions to the bottom of the tube.

COMPONENTS	20 µl REACTION	FINAL CONCENTRATION
Circular DNA Template	up to 10 µl	Variable
phi29-XT Reaction Buffer, 5X	4 µl	1X
Deoxynucleotide (dNTP) Solution Mix, 10 mM	2 µl	1 mM
Exonuclease-Resistant Random Primers, 500 µM	2 µl	50 µM
Nuclease-free Water	to 18 µl	N/A

2. Incubate in a thermocycler, with the lid set at >100°C, for 3 minutes at 95°C. Then allow samples to cool to room temperature.
3. Place samples on ice and add 2 µl of phi29-XT DNA Polymerase to each sample. Mix thoroughly, but gently, by pipetting or vortexing. Centrifuge briefly to collect solutions to the bottom of tubes.
4. Incubate in a thermocycler with the lid set at ≥ 75°C, for 2 hours at 42°C, followed by 10 minutes at 65°C to inactivate the DNA polymerase. The RCA products can be kept at 4°C overnight or at -20°C for long term storage.

   Note: The RCA products may be viscous due to the high yield of high molecular weight DNA. A two-fold dilution with nuclease-free water is recommended before use in any downstream applications.

   Note: If sample cleanup is necessary, SPRI® beads are recommended, following the manufacturer's protocol. Typically, RCA products can be directly used in downstream applications, such as Sanger sequencing, restriction digestion, and cell-free protein expression without cleanup.

Section II. phi29-XT RCA of plasmid DNA directly from bacterial cells

Input Material: Bacterial cells from agar plate colony, liquid media culture, or glycerol stock. This protocol is designed for mid- to high-copy number plasmids (> 10 copies per cell).

1. **From bacterial colony:** Using a pipette tip, pick a bacterial colony from an agar plate. Resuspend it in 10 µl nuclease-free water in a thin-walled, nuclease-free PCR tube. Optional: Save 5 µl of bacterial suspension for liquid culture or restreak it on a new agar plate.

   From liquid culture or glycerol stock: Transfer 1 µl of bacterial culture or glycerol stock to 9 µl nuclease-free water.
2. To lyse cells, incubate in a thermocycler, with the lid set at > 100°C, for 3 minutes at 95°C. Then transfer samples to ice.
3. Prepare reactions as described below. Mix thoroughly, but gently, by pipetting or vortexing. Centrifuge briefly to collect solutions to the bottom of the tube.

COMPONENTS	20 µl REACTION	FINAL CONCENTRATION
Lysed cells	up to 10 µl	N/A
phi29-XT Reaction Buffer, 5X	4 µl	1X
Deoxynucleotide (dNTP) Solution Mix, 10 mM	2 µl	1 mM
Exonuclease-Resistant Random Primers, 500 µM	2 µl	50 µM
phi29-XT DNA Polymerase, 10X	2 µl	1X
Nuclease-free water	to 20 µl	N/A

4. Incubate in a thermocycler with the lid set at ≥ 75°C, for 2 hours at 42°C, followed by 10 minutes at 65°C to inactivate the DNA polymerase. The RCA products can be kept at 4°C overnight or at -20°C for long term storage.

   Note: The RCA products may be viscous due to the high yield of high molecular weight DNA. A two-fold dilution with nuclease-free water is recommended before use in any downstream applications.

   Note: If sample cleanup is necessary, SPRI beads are recommended, following the manufacturer's protocol. Typically, RCA products can be directly used in downstream applications, such as Sanger sequencing, restriction digestion, and cell-free protein expression without cleanup.

General Protocols for Downstream Applications

Sanger Sequencing Sample Preparation

1. Dilute RCA products 5- to 20-fold with nuclease-free water.
2. Use 1 µl of diluted product to prepare Sanger sequencing samples according to sequence provider's instructions.

Cell-free Protein Expression from RCA Products Protocol

1. Dilute RCA products 20-fold with nuclease-free water.
2. Use 12 µl diluted RCA products in a 50 µl NEBExpress® Cell-free *E. coli* Protein Synthesis System (NEB #E5360) reaction, as directed in the product manual.

   Note: Protein expression can be verified by mixing 2 µl of the expression reaction with 6 µl Blue Protein Loading Dye (NEB #B7703) and 10 µl water followed by SDS-PAGE.

Debranching Protocol

1. Dilute RCA products two-fold with nuclease-free water.
2. Purify RCA products using 0.6X SPRI beads following manufacturer's recommendations.
3. Prepare debranching reactions as described below. Mix well by pipetting, and centrifuge briefly to collect solutions to the bottom of the tube.

COMPONENTS	30 µl REACTION	FINAL CONCENTRATION
Purified RCA products	Variable	Variable
NEBuffer 2, 10X	3 µl	1X
T7 Endonuclease I	1.5 µl	0.5 units/µl
Nuclease-free water	to 30 µl	N/A

4. Incubate for 1 hour at 37°C. Debranched product can be further purified by SPRI beads cleanup following manufacturer's protocol.

Troubleshooting Guide

Note: For additional assistance please refer to product FAQ's at www.neb.com/E1603.

PROBLEM	POSSIBLE CAUSE(S)	SOLUTION(S)
Low product yield	Improper reaction setup	• Perform a positive control reaction using a verified plasmid, such as pUC19 Vector (NEB #N3041).
	Inhibitor in starting material	• Chemicals in the starting material may inhibit the RCA reactions. Decrease the inhibitor concentration by diluting the starting material in water. • Purify the samples using SPRI beads or a Monarch® PCR & DNA Cleanup Kit (NEB #T1030).
	Poor DNA quality	• RCA requires an intact, circular template strand. Highly damaged or nicked DNA disrupts RCA. Use fresh DNA or repair DNA with NEB PreCR® Repair Mix (NEB #M0309).
	Low amount of input material	• Increase the amount of input material or the reaction time.
Nonspecific product bands	Contamination present in the reagents or process	• phi29-XT RCA is highly sensitive and efficient. Consequently, be mindful that small amounts of contaminating DNA may also get amplified with your sample. • Follow good laboratory PCR practices • Replace the contaminated reagent(s)
	Poor DNA integrity	• Ensure the input DNA is circular

Ordering Information

NEB #	PRODUCT	SIZE
E1603S/L	phi29-XT RCA Kit	100/500 reactions

COMPANION PRODUCTS

NEB #	PRODUCT	SIZE
E5360S/L	NEBExpress Cell-free *E. coli* Protein Synthesis System	10/100 reactions
M0302S/L	T7 Endonuclease I	250/1,250 units
N3041S/L	pUC19 Vector	50/250 µl
M0309S/L	PreCR Repair Mix	30/150 reactions
B7703S	Blue Protein Loading Dye	8 ml
N0447S/L	Deoxynucleotide (dNTP) Solution Mix	8/40 µmol
M0269S/L	phi29 DNA Polymerase	250/1,250 units
T1030S/L	Monarch DNA & PCR Cleanup Kit (5 µg)	50/250 preps

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	N/A

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