Rolling Circle Amplification (RCA) Kit without primers

AMPLIFICATION

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Introduction

TempliPhi™ amplification products from Cytiva, based on phi29 DNA polymerase, are widely used in genomics research. As the science of genomics has advanced, researchers are increasingly utilizing TempliPhi™ amplification kits in a host of new applications.

Researchers have found the ability of rolling circle amplification (RCA) to create large amounts of DNA from small amounts of input to be useful in both plant and animal studies. These researchers extensively reference TempliPhi™ amplification products for enrichment of viral genomes, amplification of complete mitochondrial genomes, and whole-community genome amplification for complex metagenomic studies. The ability of this cell-free technology to generate sufficient DNA template to produce mRNA is promising. It has the potential to generate up to 10 g of mRNA, which is the amount needed for personalized vaccine use.

To offer greater flexibility in these novel applications, we have developed a revised formulation of the TempliPhi™ Amplification Kit that does not contain any primers. The Rolling Circle Amplification (RCA) Kit without primers allows the researcher to perform specific rolling circle amplification by adding custom primers to better target their sequence of interest. The revised kit consists of 1.25x concentrated sample buffer, reaction buffer, phi29 DNA polymerase, and buffer M. The concentrated sample buffer is designed to allow enough volume for the addition of template-specific primers into the reaction. Here, we demonstrate how this revised formulation can generate high-quality DNA by rolling circle amplification that is suitable as template for transcription into functional mRNA and translation to functional protein. The DNA can be scaled up to 100 mL without additional template input.



Fig 1. Rolling Circle Amplification (RCA) Kit includes enzymes, reaction and sample buffers.

Method

Rolling circle amplification reactions with specific primers were carried out at volumes from 20 µL to 100 mL. All reactions were set up using 10 ng of starting circular template and 20 µM working concentration of template-specific forward and reverse primers. Note that heating steps are adjusted to compensate for differing reaction volumes as displayed in Table 1.

Table 1. Altered reaction conditions for scaled reactions

Reaction scale	Denaturation at 95°C	Incubation at 30°C	Heat denaturation at 65°C
10–100 µL	3 minutes	4 hours	20 minutes
1 mL	8 minutes	Overnight (18 hours)	30 minutes
10 mL	13 minutes	Overnight (18 hours)	30 minutes
100 mL	25 minutes, 400 rpm shaker	24 hours	40 minutes

Results

Scalability of RCA reaction

Following completion of RCA reactions with the RCA Kit, DNA concentrations were measured using Qubit dsDNA broad range assay kits (Thermo Fisher Scientific), and reaction yields were subsequently calculated. Scaling of the RCA reaction results in a proportional increase in dsDNA yield (Fig 2).

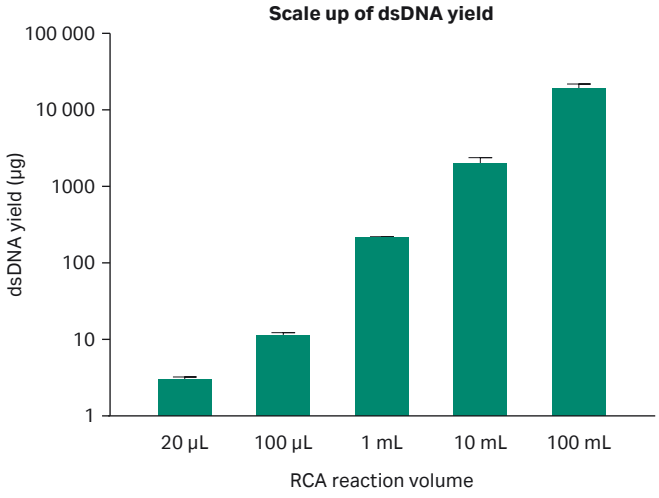


Fig 2. Total dsDNA yield of rolling circle amplification products with increasing reaction volumes. The *y*-axis is displayed in a logarithmic scale. The results are displayed as a mean, with error bars indicating standard deviation of the mean (*n* = 2–3).

Transcription of RCA products

Following DNA amplification, RCA reaction products and plasmid control were fully linearized using PmeI restriction enzyme (New England Biolabs, Inc). All linearized dsDNA was subsequently purified using Sera-Mag™ Select size selection and PCR clean-up reagent (Cytiva), and 0.5 µg of DNA from each was input into separate transcription reactions using MEGAscript transcription kits (Thermo Fisher Scientific). Following DNase treatment with TURBO DNase (Thermo Fisher Scientific), post transcription products were cleaned up using MEGAclear kits (Thermo Fisher Scientific), and concentrations of mRNA from each transcription were measured using Qubit broad range RNA kits (Thermo Fisher Scientific). Diluted mRNAs were also run on the 2100 Bioanalyzer (Agilent Technologies, Inc.) using the RNA nano reagents kit and chip (Agilent Technologies, Inc.).

Yields of mRNA generated from transcription reactions remain consistent irrespective of the scale from which the input DNA is derived (Fig 3A). Additionally, all the mRNA profiles overlap and are comparable to those directly transcribed from the plasmid controls, indicating that RCA scale up has no effect on mRNA transcription (Fig 3B).

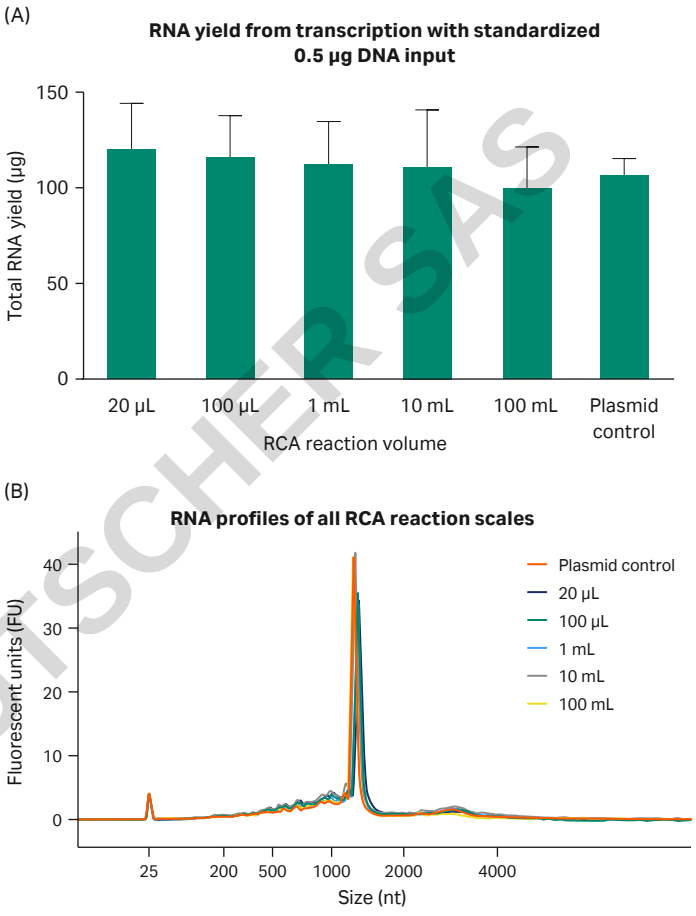


Fig 3. RNA transcript yields and transcription products are equivalent across samples from all reaction scales. (A) RNA transcript yields of RCA products and plasmid control are displayed as average total yields in µg (*n* = 2–3). Error bars indicate standard deviation of the mean. One-way analysis of variance (ANOVA) shows no significant difference in RNA yield across samples ($p = 0.9081$). (B) RNA profiles obtained from the 2100 Bioanalyzer, with samples from one transcription experimental repeat overlayed. The key shows the color codes of samples.

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Sequencing of RCA products

A comparison of DNA sequences of RCA product vs plasmid was performed by next generation sequencing (NGS). Samples were generated in RCA reactions with the RCA Kit at 20 µL and 100 mL scales. RCA products and plasmid controls were linearized with restriction enzyme and cleaned using Sera-Mag™ Select size selection and PCR clean-up reagent (Cytiva). Samples were sent externally for sequencing. Analysis included mapping against predicted plasmid sequences, and results were given as percentage of mapped reads.

Results show that at both a low reaction volume (20 µL) and a high reaction volume (100 mL), all RCA product DNA sequences had a highly comparable percentage of mapped reads to the plasmid control (Fig 4).

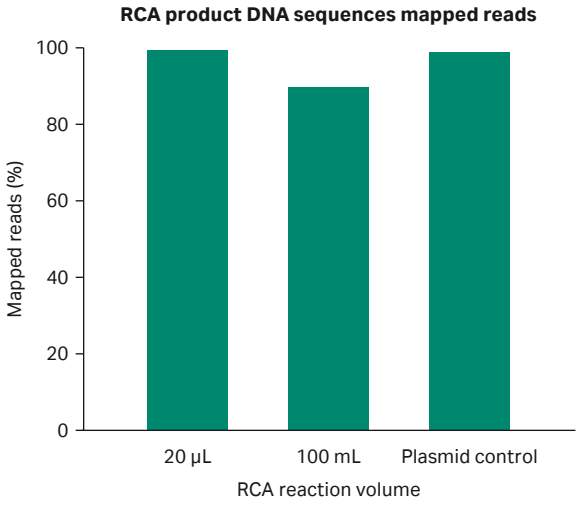


Fig 4. Percentage mapped reads of RCA product DNA sequences at low- and high-volume reaction scales compared to plasmid control DNA. Sequences were compared to predicted plasmid sequences.

Translation of RCA product

Following transcription, 1 ug of mRNA transcripts of 100 mL RCA products were input into translation reactions alongside plasmid control mRNA (Thermo Fisher Scientific). The plasmid template contained the coding sequence of enhanced green fluorescence protein (EGFP). Therefore, the translation of functional protein was assessed by green fluorescence output, measured in relative fluorescence units (RFU), and normalized to the fluorescence of the plasmid control translation products. Results show effective fluorescence of protein translated from RCA product compared to plasmid control.

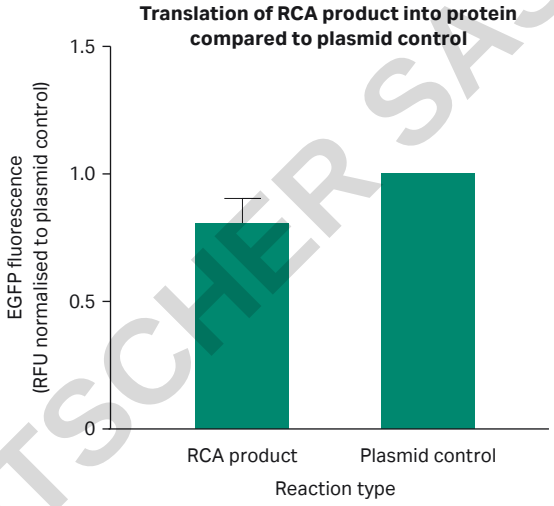


Fig 5. Equivalent fluorescence of functional EGFP protein from translation of RCA products and plasmid control. Fluorescence is displayed in relative fluorescence units (RFU) and are standardized to plasmid controls.

Ordering information

Product	Pack size	Product code
Rolling Circle Amplification (RCA) Kit without primers	N/A	Contact modality specialist
TempliPhi™ 100/500 Amplification Kits	100 reactions 500 reactions	25640010 25640050
TempliPhi™ 2000 Amplification Kits	2000 reactions	28964286
TempliPhi™ Large Construct Kit	1000 reactions	25640080
TempliPhi™ Sequence Resolver Kit	20 reactions 200 reactions	28903529 28903531
Sera-Mag™ Select size selection and PCR clean-up reagent	5 mL 60 mL 450 mL 20 × 5 mL	29343045 29343052 29343057 29453302

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