Isothermal amplification

From DNA construction to protein in 1 day

Cell-free workflow combining Gibson Assembly cloning or self-circularization with rolling circle amplification using the EquiPhi29 DNA Amplification Kit

Key findings

- Thermo Scientific™ EquiPhi29™ DNA Polymerase amplifies DNA directly from assembly reactions, eliminating the need for template isolation
- Rolling circle amplification reaction product can be used for efficient cell-free protein expression
- A streamlined, cell-free process for high-throughput protein engineering is demonstrated

Introduction

Cloning genes and expressing the corresponding proteins is a multiday and labor-intensive process that relies on prokaryotic or eukaryotic cells. To reduce time and labor, a cell-free protocol that combines Gibson Assembly™ cloning or gene-of-interest self-circularization, rolling circle amplification (RCA), and cell-free protein expression (CFPE) may be feasible (Figure 1). This protocol omits traditional cloning steps such as transformation and plasmid purification. The workflow allows construction of a genetic template, amplification, and protein expression within a single day. In comparison, traditional protein expression methods require at least 2 days of experimental work or more steps.

To clone a gene of interest, the Invitrogen™ GeneArt™ Gibson Assembly™ HiFi Master Mix can be used. It enables DNA assembly via a technique that allows overlapping DNA fragments to be seamlessly linked (with no extra sequences and scars) in one isothermal reaction. DNA fragments of different lengths are uniformly assembled using complementary overlaps between fragments resulting in the covalently bound final product. Traditionally, bacterial cells are transformed with these reaction products, and the plasmid is purified from the selected positive clones. As the product of Gibson Assembly cloning is circular, it can be amplified by RCA, a highly efficient isothermal amplification method for circular nucleic acids. A strand-displacing polymerase such as EquiPhi29 DNA Polymerase generates high yields of long concatemeric products in just 2 hours from a small amount of template. These products can be used in various downstream applications such as sequencing, cell-free protein expression, and restriction digestion.

Alternatively, the gene of interest with regulatory sequences (promoter, ribosome-binding site, terminator) can be self-circularized via ligation using the Thermo Scientific™ Rapid DNA Ligation Kit and subsequently used as RCA template for DNA amplification. Thus, an array of different gene variants can be rapidly amplified and used in functional tests for high-throughput selection of gene variants. This method may further reduce costs compared to Gibson Assembly cloning. It is important to note that this approach eliminates the need for vector cloning, but the construct is not suitable for protein expression in host systems that require specific vector elements.

Here we demonstrate various applications including restriction digestion, Sanger sequencing, and cell-free protein expression using a template amplified via RCA with the Thermo Scientific™ EquiPhi29™ DNA Amplification Kit directly from Gibson Assembly cloning or self-circularization reactions. This cell-free workflow reduces experimental time from ~3 days to ~1 day by replacing time-consuming steps of bacterial transformation, liquid culture, and plasmid purification that are required in traditional cloning workflows with a fast and efficient RCA reaction (Figure 1).

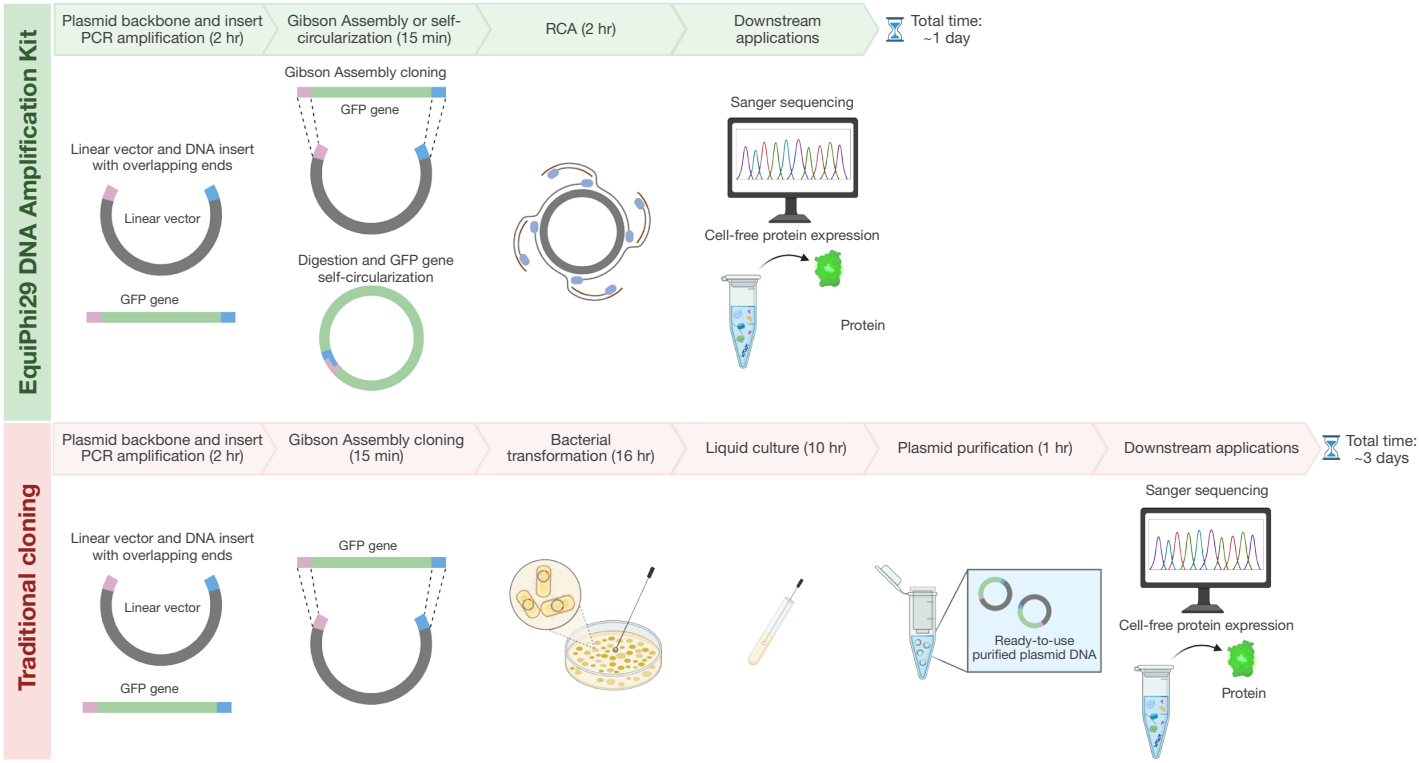

Figure 1. Comparison of total reaction time using a combination of Gibson Assembly cloning or self-circularization and RCA versus the traditional cloning approach. The cell-free workflow reduces experimental time from ~3 days to ~1 day by omitting time-consuming bacterial transformation, liquid culture, and plasmid purification steps and replacing them with a fast, efficient RCA reaction. Created with BioRender.com.

Materials and equipment

- Thermo Scientific™ pUC19 DNA (Cat. No. SD0061)
- Invitrogen™ GeneArt™ Strings™ DNA Fragment (GFP gene)
- Oligonucleotides for PCR and Sanger sequencing (Table 1)
- Invitrogen™ Platinum™ SuperFi™ II DNA Polymerase (Cat. No. 12361010)
- Thermo Scientific™ dNTP Mix (10 mM each) (Cat. No. R0191)
- Thermo Scientific™ GeneJET™ Gel Extraction and DNA Cleanup Micro Kit (Cat. No. K0832)
- Invitrogen™ GeneArt™ Gibson Assembly™ HiFi Master Mix (Cat. No. A46628)
- Invitrogen™ One Shot™ TOP10 Chemically Competent *E. coli* (Cat. No. C404003)
- Thermo Scientific™ GeneJET™ Plasmid Miniprep Kit (Cat. No. K0503)
- Thermo Scientific™ FastDigest™ KpnI (Cat. No. FD0524)
- Thermo Scientific™ FastDigest™ DpnI (Cat. No. FD1703)
- Thermo Scientific™ Rapid DNA Ligation Kit (Cat. No. K1422)
- Thermo Scientific™ EquiPhi29™ DNA Amplification Kit (Cat. No. A65393)
- Invitrogen™ Quant-iT™ PicoGreen™ dsDNA Assay Kits and dsDNA Reagents (Cat. No. P11495)
- Biotechrabbit™ RTS 100 *E. coli* HY Kit (Biotechrabbit, Cat. No. BR1400101)
- Thermo Scientific™ Water, nuclease-free (Cat. No. R0581)
- Invitrogen™ E-Gel™ 48 Agarose Gels with SYBR™ Safe DNA Gel Stain, 1% (Cat. No. G820841)
- Invitrogen™ E-Gel™ 1 Kb Plus Express DNA Ladder (Cat. No. 10488091)
- Invitrogen™ E-Gel™ Power Snap Plus Electrophoresis System (Cat. No. G9101)
- Invitrogen™ E-Gel™ EX Agarose Gels, 1% (Cat. No. G401001)
- Thermo Scientific™ 96 Black Plate, Non-Treated Coverglass Surface (Cat. No. 265300)
- Invitrogen™ Luria Broth Base (Miller's LB Broth Base), powder (Cat. No. 12795027)
- Applied Biosystems™ ProFlex™ PCR System (Cat. No. 4484073)
- Thermo Scientific™ NanoDrop™ One^{C} Spectrophotometer (Cat. No. ND-ONEC-W)
- Thermo Scientific™ Varioskan™ LUX Multimode Microplate Reader (Cat. No. VLBLATD1)

See ordering information on last page for more information.

Table 1. Oligonucleotide sequences.

Name	5´ to 3´ sequence	Description
GFP_Fw	CTG CAA GGC GAT TAA GTT GG	Forward primer for GFP insert amplification
GFP_Rv	CAG GAA ACA GCT ATG ACC AT	Reverse primer for GFP insert amplification
pUC19_Fw	TGG CGT AAT CAT GGT CAT AGC	Forward primer for pUC19 backbone amplification
pUC19_Rv	CTG GCG TTA CCC AAC TTA ATC	Reverse primer for pUC19 backbone amplification
Primer 1	CAT TCG CCA TTC AGG CTG CG	Forward colony PCR primer, sequencing primer
Primer 2	GAT AAC ACT GCG GCC AAC TT	Reverse colony PCR primer
Primer 3	ACT GGA AAG CGG GCA GTG AG	Sequencing primer
Primer 4	TAA TAC GAC TCA CTA TAG G	Sequencing primer

Methods

Preparation of DNA fragments for assembly reactions

The gene sequence encoding green fluorescent protein (GFP) from *Azotobacter vinelandii* was adapted for protein expression in *E. coli* using the Invitrogen™ GeneArt™ GeneOptimizer™ algorithm and ordered as a GeneArt Strings DNA Fragment containing a ribosome binding site, a T7 promoter at the 5′ end, and a T7 terminator at the 3′ end. To facilitate Gibson Assembly cloning, 30 bp regions overlapping with pUC19 destination vector were added to each end of the DNA fragment. The synthesized GFP gene fragment and pUC19 backbone were amplified by PCR to obtain linear fragments with overlaps at the ends suitable for the Gibson Assembly cloning reaction. The amplification was performed using high-fidelity Platinum SuperFi II DNA Polymerase according to recommended conditions using GFP_Fw and GFP_Rv primers for GFP amplification, and pUC19_Fw and pUC19_Rv primers for pUC19 backbone amplification. Following DNA amplification, vector DNA was treated with 2.5 µL of FastDigest DpnI at 37°C for 15 min to remove the initial pUC19 vector. Upon reaction completion, both amplified vector backbone and GFP DNA fragments were purified and concentrated using the GeneJET Gel Extraction and DNA Cleanup Micro Kit according to the PCR cleanup and dimers removal protocol. DNA concentration was measured using the NanoDrop One^{C} Spectrophotometer, and the quality of prepared fragments was verified by analysis on precast 1% E-Gel EX Agarose Gels according to recommendations.

Gibson Assembly cloning

Gibson Assembly cloning was carried out using the GeneArt Gibson Assembly HiFi Master Mix according to the recommendations in the user guide. In brief, the vector backbone and GFP fragments were mixed at a molar ratio of 1:1. DNA assembly was performed in a total volume of 20 µL containing 0.08 pmol of each DNA fragment and 10 µL of GeneArt Gibson Assembly HiFi Master Mix. Samples were incubated in a thermal cycler at 50°C for 15, 30, or 45 min. The assembled constructs were directly used in the RCA reaction or in a traditional cloning protocol.

Self-circularization

The amplified GFP fragment was digested with FastDigest KpnI at 37°C for 5 min, and the enzyme was inactivated by incubation at 80°C for 5 min. Then, 1 ng/µL of digested GFP gene was self-ligated using the Rapid DNA Ligation Kit at 20°C for 5 min followed by heat-inactivation at 70°C for 5 min. Self-circularized GFP was directly used in the RCA reaction.

Traditional cloning (transformation, colony validation, plasmid prep)

One Shot TOP10 Chemically Competent *E. coli* cells were transformed with 1 µL of 1:5 diluted Gibson Assembly reaction products according to recommendations provided in the GeneArt Gibson Assembly HiFi Master Mix user guide. 100 µL from each transformation was spread onto pre-warmed selective Luria-Bertani (LB) agar plates containing 100 µg/mL ampicillin and incubated overnight at 37°C. Isolated colonies were subsequently used for verification by colony PCR using primers 1 and 2, and Platinum SuperFi II DNA Polymerase according to instructions. The representative clones were subsequently picked and used to start 4 mL cultures in LB ampicillin broth. These *E. coli* cultures were grown overnight at 37°C, 200 rpm. The plasmids from different clones were purified using the GeneJET Plasmid Miniprep Kit according to recommendations. Purified plasmids were submitted for clone verification by Sanger sequencing using primers 1 and 3 targeting vector DNA flanking GFP and vector junction sites.

Rolling circle amplification (RCA)

Purified pUC19-GFP plasmid, picked single colony, Gibson Assembly reaction mix, and self-circularization reaction mix were used as templates in RCA reaction mixes using the EquiPhi29 DNA Amplification Kit. The reaction mixes without enzymes were prepared as described in Table 2 and subjected to heat treatment in a thermal cycler at 95°C for 3 minutes to denature DNA. Afterward, heated mixes with samples were placed on ice, and 1 µL of EquiPhi29 DNA Polymerase and 1 µL of pyrophosphatase were added. The reaction was allowed to proceed at 42°C for 2 hours. Then, the reaction was heated for 10 minutes at 65°C to heat inactivate the polymerase. The yield of RCA reactions was quantified using the Quant-iT PicoGreen dsDNA Assay Kit according to instructions while the quality was assessed by analysis of intact and digested RCA products on an E-Gel agarose gel. Briefly, 1 µL of RCA product was digested with FastDigest KpnI in a 20 µL reaction at 37°C for 5 min followed by heat inactivation at 80°C for 5 min. 2 µL of digested and 20x diluted intact RCA product was analyzed on a 1% E-Gel EX Agarose Gel. Additionally, 20x diluted non-digested RCA products were analyzed by Sanger sequencing using primer 4.

Cell-free protein expression

Cell-free protein expression was carried out using 1 µL of RCA products or 500 ng purified pUC19-GFP plasmid in a reaction volume of 50 µL with the Biotechrabbit RTS 100 *E. coli* HY Kit according to the manufacturer's recommendations. The reactions were incubated at 30°C overnight in a 96 Black Plate on a Varioskan LUX Multimode Microplate Reader. The protein synthesis was monitored by fluorescence measurement with an excitation wavelength of 395 nm and an emission wavelength of 504 nm (with a 5 nm bandwidth) every 5 minutes. The plate was shaken for 5 seconds at 180 rpm before each measurement.

Table 2. RCA reaction setup using EquiPhi29 DNA Amplification Kit (20 µL reaction).

Component	20 µL reaction	Final composition
10X EquiPhi29 DNA Polymerase Reaction Buffer	2 µL	1X
100 mM DTT	0.2 µL	1 mM
500 µM Exo-Resistant Random Primer	2 µL	50 µM
10 mM dNTP Mix	2 µL	1 mM each
Template	1 µL	1 µL of one of the following: • Single colony resuspended in 10 µL of nuclease-free water • 1 ng/µL purified pUC19-GFP • 10x diluted Gibson Assembly reaction product • Self-circularization reaction product • Nuclease-free water as non-template control (NTC)
Nuclease-free water	Add to 18 µL volume	–
Add after heating step:		
EquiPhi29 DNA Polymerase	1 µL	0.5 U/µL
Pyrophosphatase	1 µL	0.005 U/µL

Results

Gibson Assembly reaction time course

GFP reporter from *A. vinelandii* was selected for cell-free protein expression analysis using a template amplified via RCA. First, Gibson Assembly reaction efficiency with GeneArt Gibson Assembly HiFi Master Mix was tested by incubating pUC19 backbone and GFP insert in reaction mix at different times (15, 30, or 45 minutes). Later, assembly mixes were used for *E. coli* transformation, and the colony forming units (CFU) counted. Additionally, an aliquot of respective Gibson Assembly reaction was used in RCA. The results show that independent of Gibson Assembly reaction time, similar CFU counts and RCA reaction yields were achieved (Figure 2A and 2B). To verify accuracy of the Gibson Assembly reaction, 14 randomly selected clones for each time point were screened by colony PCR. All tested clones were verified to have an insert (Figure 2C) while the correct sequence was confirmed by Sanger sequencing for 15 out of 15 analyzed clones. Thus, a 15-minute Gibson Assembly reaction is recommended for a quick protocol.

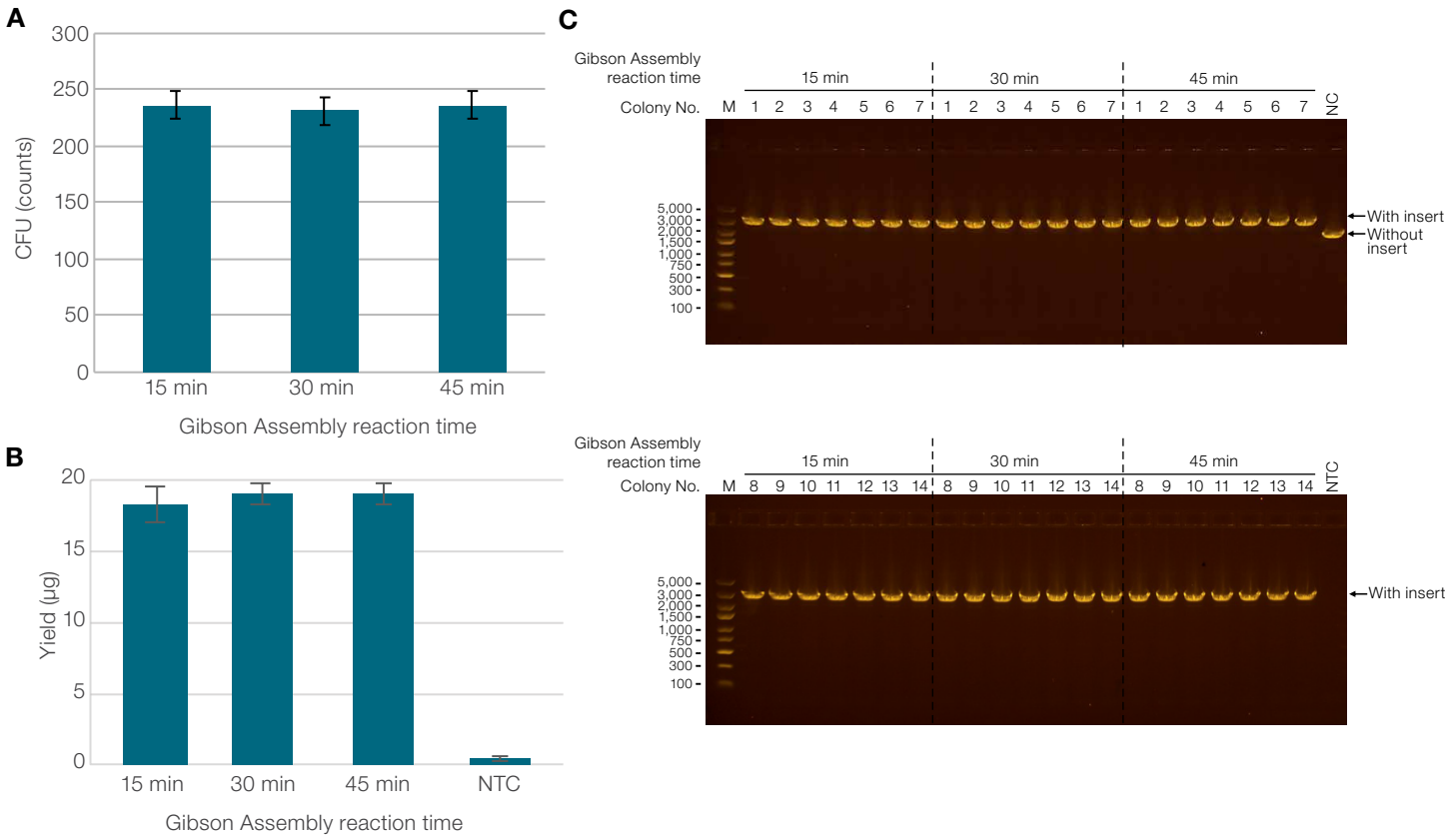

Figure 2. Dependence of Gibson Assembly reaction efficiency and accuracy on reaction time. (A) Comparison of colony formation of *E. coli* transformed with Gibson Assembly reaction mixtures. Data represent the average CFU counts with standard deviation (n = 9). **(B)** RCA reaction yields using Gibson Assembly cloning products as a template. Reaction yields were quantified using the Quant-iT PicoGreen dsDNA Assay Kit. Data represent the mean with standard deviation (n = 3). **(C)** Colony PCR screening of *E. coli* transformants using vector-specific primers flanking the insert. Equal amounts of PCR product were loaded on a 1% E-Gel agarose gel with SYBR Safe dye. M: E-Gel 1 Kb Plus Express DNA Ladder used as a molecular size standard, NC: negative control consisting of vector without insert, NTC: non-template control.

RCA efficiency using different templates

To test whether the RCA reaction can be carried out using different templates, 4 template types were prepared: (1) self-circularized GFP gene, (2) Gibson Assembly reaction sample with pUC19 backbone and GFP insert, (3) *E. coli* transformed with Gibson Assembly reaction products, and (4) purified pUC19-GFP plasmid. RCA reactions were performed at 42°C for 2 hours with 1 ng of input DNA from self-circularization or Gibson Assembly reaction mixtures, a single resuspended *E. coli* transformant, 1 ng of purified plasmid, or no template (NTC). High DNA yield after RCA was obtained from all tested DNA templates reaching up to ~18 µg (Figure 3A). To further assess the success and specificity of the reaction, the RCA products were analyzed using an E-Gel agarose gel (Figure 3B). The analysis included intact samples and those digested with KpnI, which cuts at the ends of GFP inserts in a site-specific manner. In the case of non-digested samples, a large amount of high molecular weight products was detected as a smear above the purified plasmid control confirming the generation of RCA product. NTC did not give any visible signal, indicating the absence of RCA product. The length of the main KpnI-digested RCA products matched the control bands corresponding to vector backbone or GFP insert. RCA amplicon from GFP self-circularization produces a single band that corresponds to GFP insert because there was no plasmid backbone fragment in the self-circularization reaction.

These results confirm that the EquiPhi29 DNA Amplification Kit delivers high yields of DNA products from various starting materials including Gibson Assembly cloning reactions, self-circularization reaction mixtures, and bacterial cells. Moreover, EquiPhi29 DNA Polymerase was not inhibited by side-products of assembly reactions indicating its potential to be used for various DNA applications.

Sanger sequencing and cell-free protein expression using RCA products as templates

To verify the sequence of RCA amplicons, RCA products and plasmid extracted from bacterial culture were analyzed by Sanger sequencing. The chromatograms of all samples were of high quality, and the length of high-quality traces was similar between samples ranging from 924 nt to 1,030 nt (Figure 4A).

Following sequence verification, a functional test of RCA products was performed via cell-free protein expression. 1 µL of RCA amplicons or 500 ng of purified plasmid was used for protein synthesis with the RTS 100 *E. coli* HY Kit. Figure 4B illustrates that all experimental samples showed similar strong fluorescent signals whereas no signal was observed when RCA negative control was used (NTC). This highlights that RCA products enable cell-free protein expression of GFP as efficiently or even better than purified plasmid. Interestingly, RCA product from self-circularized GFP results in the same protein expression level as RCA products from Gibson Assembly reaction or purified plasmid. Thus, the gene of interest can be self-circularized using the Rapid DNA Ligation Kit without the need for a plasmid backbone, simplifying the workflow, increasing throughput capacity, and reducing cost.

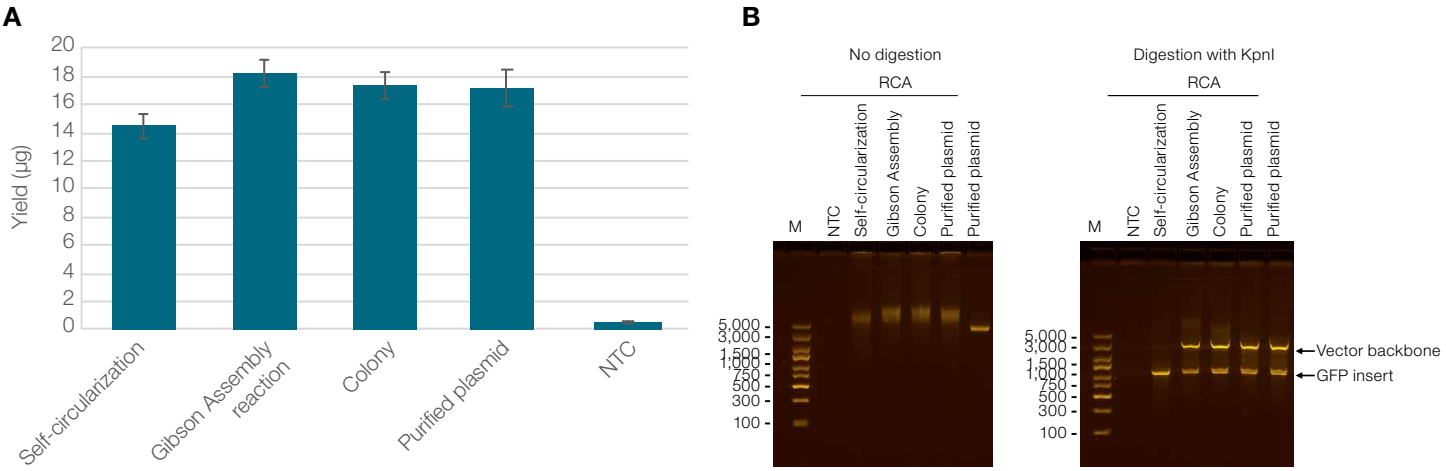

Figure 3. Quantity and quality of RCA products from different templates. (A) Yield of RCA directly from self-circulization, Gibson Assembly reaction, single colony, or purified plasmid. Data show the mean value ± standard deviation (n = 3). **(B)** Agarose gel electrophoresis of non-digested (left) or restriction-digested (right) RCA products.

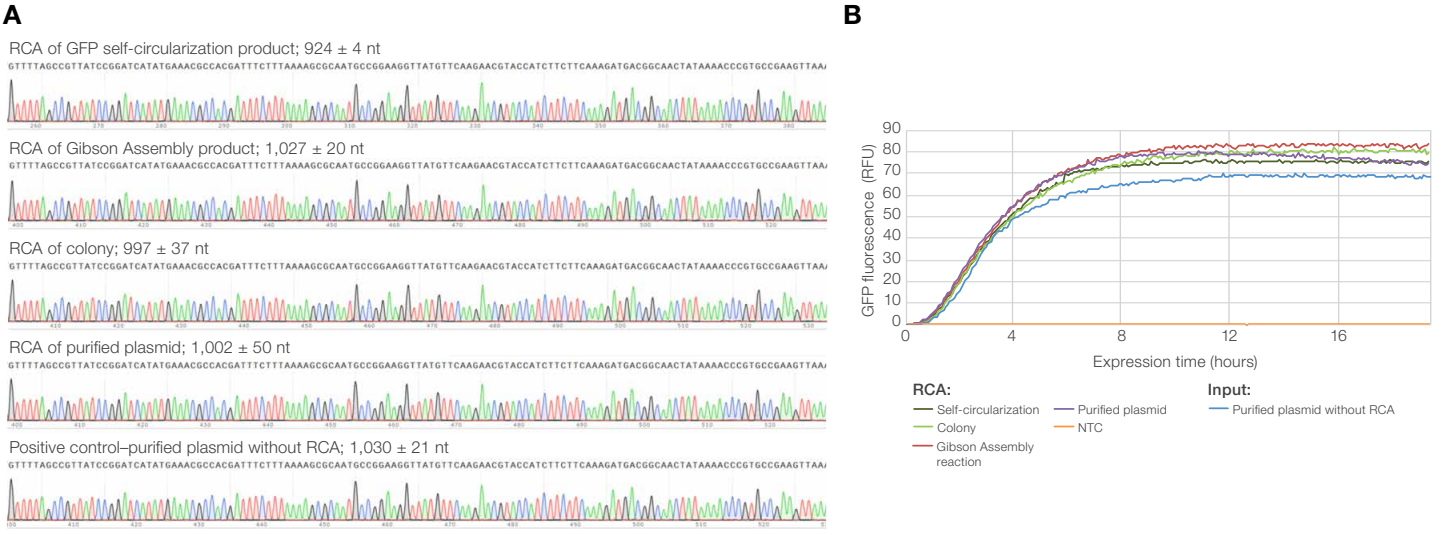

Figure 4. Comparison of Sanger sequencing quality and protein expression level from RCA products amplified using different templates. (A) Sanger sequencing chromatograms of RCA products and non-amplified plasmid. The numbers indicate the average length of high-quality trace with standard deviation (n = 3). **(B)** Time course of GFP synthesis during the cell-free protein expression reaction using different DNA inputs.

Conclusions

By combining the EquiPhi29 DNA Amplification Kit with either the GeneArt Gibson Assembly HiFi Master Mix or the Rapid DNA Ligation Kit along with a cell-free protein expression system, functional protein can be made in just 1 day starting from DNA fragments. This method saves several steps and reduces experimental time by up to 2 days when compared to traditional cloning protocols.

Ordering information

Product	Quantity	Cat. No.
pUC19 DNA	50 µg	SD0061
Platinum SuperFi II DNA Polymerase	100 reactions	12361010
dNTP Mix (10 mM each)	0.2 mL	R0191
GeneJET Gel Extraction and DNA Cleanup Micro Kit	250 preps	K0832
GeneArt Gibson Assembly HiFi Master Mix	50 reactions	A46628
One Shot TOP10 Chemically Competent *E. coli*	21 x 50 µL/tube	C404003
GeneJET Plasmid Miniprep Kit	250 preps	K0503
FastDigest KpnI	300 reactions	FD0524
FastDigest DpnI	50 reactions	FD1703
Rapid DNA Ligation Kit	50 reactions	K1422
EquiPhi29 DNA Amplification Kit	100 reactions	A65393
Quant-iT PicoGreen dsDNA Assay Kits and dsDNA Reagents	10 x 100 µL	P11495
Water, nuclease-free	4 x 1.25 mL	R0581
E-Gel 48 Agarose Gels with SYBR Safe DNA Gel Stain, 1%	4 x 8 gels	G820841
E-Gel 1 Kb Plus Express DNA Ladder	100 applications	10488091
E-Gel Power Snap Plus Electrophoresis System	1 device	G9101
E-Gel EX Agarose Gels, 1%	10 gels	G401001
96 Black Plate, Non-Treated Coverglass Surface	Case of 30	265300
Luria Broth Base (Miller's LB Broth Base), powder	500 g	12795027
ProFlex PCR System	1 instrument	4484073
NanoDrop One^{C} Spectrophotometer	1 unit	ND-ONEC-W
Varioskan LUX Multimode Microplate Reader	1 unit	VLBLATD1

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