INSTRUCTION MANUAL

EnClose™ Cell-free dbDNA™ Synthesis Kit

NEB #E9301S

20 reactions

Version 1.0_1/26

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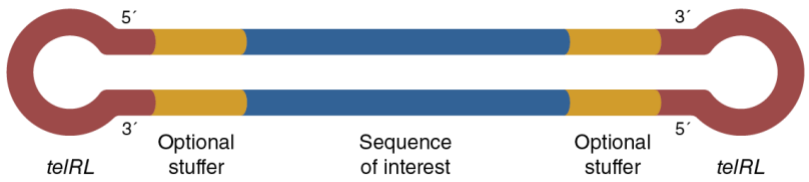
Overview

The EnClose Cell-free dbDNA Synthesis Kit includes everything needed to enzymatically generate closed-ended linear dsDNA (dbDNA, Figure 1) containing a sequence of interest (SOI) (1). The robust combination of phi29-XT DNA Polymerase for high-yield rolling circle amplification (RCA) and TelN Protelomerase for deconcatenation and covalent closure of linear dsDNA ends enables a streamlined one-day workflow (Figure 3) that produces cell-free dbDNA for downstream applications such as *in vitro* transcription (IVT), lentiviral (LV) payload, adeno-associated virus (AAV) payload and more.

Figure 1. A dbDNA is a linear, double-stranded DNA with covalently closed ends



Kit Components

The kit should be stored at −20°C upon receipt, is stable for at least 30 freeze-thaw cycles and has a shelf life of 24 months when properly stored. 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.

Table 1. Kit components and storage information

COMPONENT	NEB #	SIZE	CONCENTRATION
dbDNA Vector	N9301S	50 µl	0.5 mg/ml
phi29-XT DNA Polymerase	M0572M	0.2 ml	10X
dbDNA Reaction Buffer	B9301S	1 ml	10X
dbDNA Primer Mix	S9301S	0.2 ml	0.5 mM each
Deoxynucleotide (dNTP) Solution Mix*	N0447AA	0.8 ml	10 mM each
Pyrophosphatase, Inorganic (*E. coli*)*	M0361S	10 U	100 units/ml
EnClose TelN Protelomerase*	M0768M	0.2 ml	25,000 U/ml
XbaI*	R0145T	3,000 U	100,000 U/ml
T5 Exonuclease*	M0663S	5,000 U	10,000 U/ml

* This component is available as a GMP-grade product from NEB.

Cloning your Sequence of Interest

About the dbDNA Vector

The dbDNA Vector provided with this kit is designed to serve as both an input source for positive control reactions, and as an optimized, flexible destination vector for your own sequence of interest (SOI) upstream of dbDNA production. The dbDNA Vector utilizes a high-copy pUC origin (*ori*) for higher yields and contains the Kanamycin resistance gene (*Kan*^{R}) for reliable transformant selection following cloning.

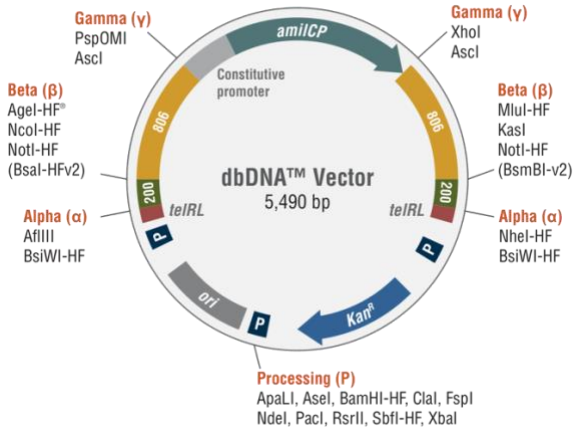
Cloning your SOI into the dbDNA Vector is uniquely flexible. It can be achieved through a variety of cloning modalities supported by New England Biolabs, including traditional restriction enzyme (RE) cloning, NEBuilder® HiFi DNA Assembly, and NEBridge® Golden Gate Assembly (for insertion of high-complexity or repetitive elements).

The inclusion of stuffer sequences is application specific. Some specific examples include:

- **AAV payload dbDNA containing highly structured ITRs** – it can be beneficial to have added space between the ITR elements and the *telRL* end of the dbDNA.
- **LV sequences** – stuffers greatly improve functionality and provide a primer binding region to facilitate sequencing.

The dbDNA Vector (Figure 2) contains a variety of restriction sites for downstream processing to give users flexibility in cloning without the requirement for specific domestication of their sequences. When selecting a restriction endonuclease for downstream processing (i.e., removal of dbDNA Vector backbone after dbDNA formation), it is important that the recognition sequence is not present in the dbDNA SOI (see Supplementary Table 1 for sequence information).

Figure 2. dbDNA Vector



Processing restriction endonucleases

XbaI is provided in this kit as the processing restriction endonuclease. Before using the included enzyme, ensure that the XbaI recognition sequence is not present in your sequence of interest (SOI). If domestication of the SOI is not possible, use one of the alternative processing restriction endonucleases listed below.

- **Alternative processing restriction endonucleases (3)** – ApaLI, AseI, BamHI-HF®, ClaI, FspI, NdeI, PacI, RsrII, SbfI-HF

Please refer to neb.com for guidance on the various cloning strategies.

Before you Begin

Required equipment and materials

- Thermocycler
- DNA quantification instrument (e.g., Nanodrop®, Qubit®, etc.)
- PCR strip tubes or 96-well plates
- Purified single- or double-stranded circular DNA (5 ng/µl) – must contain *telRL* recognition sequences flanking SOI (see Supplementary Figure S1)
- Nuclease-free water
- Recommended (*not supplied in this kit*):
  - Monarch® Spin High-Capacity DNA Cleanup Kit (100 µg) (NEB #T1135)
  - When scaling down reactions: Monarch Spin PCR & DNA Cleanup Kit (5 µg) (NEB #T1130) or Monarch Mag PCR & DNA Cleanup Kit (5 µg) (NEB #T4130)

Using dbDNA Vector in control reactions

The dbDNA Vector can be used as a positive control input for dbDNA synthesis. The *amilCP* reporter protein is cloned into dbDNA Vector with the γ strategy, retaining both upstream and downstream 200-bp and 806-bp stuffers. A successful reaction using the dbDNA Vector as an input for dbDNA synthesis will produce a 3,063-bp final product.

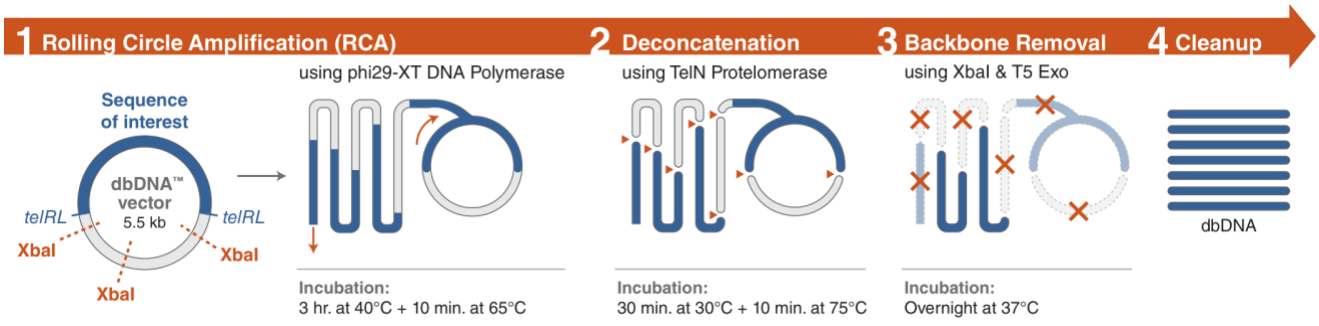
Primers for RCA

A specific primer mix is provided in this kit, consisting of a pair of forward and reverse primers that directly bind to the Kanamycin resistance gene located in the provided dbDNA Vector for cloning in your SOI. The specific primers enable targeted amplification of your RCA input plasmid and limit the amount of unwanted DNA products that can burden the restriction endonuclease and exonuclease cleanup step.

In addition, the provided primers are exonuclease-resistant with the addition of phosphorothioate bonds to protect them from degradation by phi29-XT DNA Polymerase, which exhibits strong 3′→5′ exonuclease proofreading activity.

If using different primers than those provided in this kit, it is recommended that the primers are protected with a minimum of two phosphorothioate bonds on the 3′ end.

Figure 3. Overview of the EnClose Cell-free dbDNA Synthesis Kit workflow



Protocol for dbDNA Synthesis

Part 1: Rolling Circle Amplification (RCA)

1.1. Prepare reactions on ice without polymerase, as described in the table below. Mix thoroughly by pipetting or vortexing. Centrifuge briefly to collect solutions to the bottom of the tube. We recommend diluting the input plasmid to a working concentration of 5 ng/µl.

COMPONENT	90 µl REACTION	FINAL CONCENTRATION
DNA Template (dbDNA Vector or assembly, 5 ng/µl)	2 µl	0.1 ng/µl (10 ng total input)
10X dbDNA Reaction Buffer	10 µl	1X
Deoxynucleotide (dNTP) Solution Mix	20 µl	2 mM
dbDNA Primer Mix	10 µl	50 µM
Pyrophosphatase, Inorganic (*E. coli*)	1 µl	0.001 U/µl
Nuclease-free water	to 90 µl	—

Increasing the initial circular template concentration or incubation time (Step 1.3.) may increase the RCA product yield; the latter may be required for extremely low RCA inputs (e.g., 1 fg of 2 kb plasmid).

1.2. Add 10 µl phi29-XT DNA Polymerase to each sample. Mix thoroughly, but gently, by pipetting the whole volume. Centrifuge briefly to collect solutions to the bottom of tubes.

1.3. Incubate in a thermocycler (with heated lid to prevent evaporation) at 40°C for 3 hours, followed by 10 minutes at 65°C. Allow samples to cool to room temperature before proceeding.

Note: 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 40°C. Amplification below 37°C using this kit is not recommended.

1.4. (*Optional*) Quantify your RCA product for amplification verification.

Notes:

- RCA products may be directly quantified after dilution by Quant-iT® PicoGreen® dsDNA Assay Kit or Qubit Fluorometer.
- Purified RCA products can be quantified by measuring the absorbance at 260 nm (e.g., NanoDrop), but may be less accurate due to the presence of unincorporated nucleotides and primers.
- RCA product quality can be verified by digesting with a restriction endonuclease and running the products on an agarose gel.

Safe stopping point – store samples at −20°C. Thaw at room temperature when ready to proceed.

Part 2: dbDNA Formation

2.1. At room temperature, add 8 µl EnClose TelN Protelomerase (200 U) directly to the PCR tube containing the completed RCA reaction. Mix thoroughly, but gently, by pipetting the whole volume.

The reaction should be viscous due to the high amounts of DNA from amplification.

Do NOT dilute the reaction prior to adding EnClose TelN Protelomerase. Any dilution of the reaction prior to adding EnClose TelN Protelomerase will reduce the efficiency of the protelomerase and result in reduced dbDNA yields.

Tip: Using a wide-bore pipette tip may help to achieve optimal mixing at this step.

2.2. Incubate in a thermocycler (with heated lid to prevent evaporation) at 30°C for 30 minutes, followed by 10 minutes at 75°C.

Allow samples to cool to room temperature before proceeding.

Safe stopping point – store samples at −20°C. Thaw at room temperature when ready to proceed.

Part 3: dbDNA Cleanup

3.1. Add 2 µl T5 exonuclease (20 U) and 1 µl of XbaI (100 U). Mix thoroughly, but gently, by pipetting the whole volume.

Note: An alternative processing restriction endonuclease may be used in place of XbaI. See "Processing Restriction Endonucleases" section. and Supplementary Table 1 for more information.

3.2. Incubate in a thermocycler (with heated lid to prevent evaporation) at 37°C overnight (~18 hours).

Safe stopping point – store samples at −20°C. Thaw at room temperature when ready to proceed.

3.3. dbDNA samples must be purified before use. We recommend using the Monarch Spin High-Capacity DNA Cleanup Kit (100 µg) (NEB #T1135) to remove any residual NTPs, buffers, salts, and enzymes.

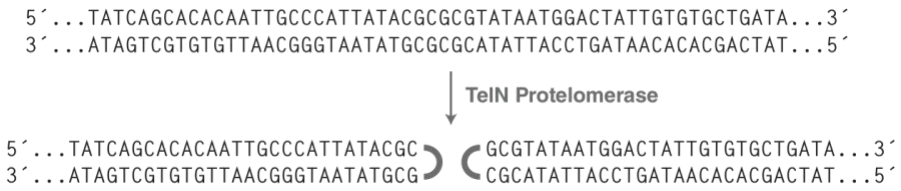
Note: If using the dbDNA vector as a control, expected yields of dbDNA are > 20 µg.

3.4. Quantify your dbDNA product to prepare for downstream applications:

- Purified dbDNA products can be quantified by measuring the absorbance at 260 nm (e.g., NanoDrop) or other microvolume spectrophotometer, such as Quant-iT PicoGreen dsDNA Assay Kit, or Qubit Fluorometer.
- dbDNA product quality can be assessed by running the purified dbDNA products on an agarose gel.

Supplementary Information

Supplementary Figure S1. TelN Protelomerase reaction mechanism at single *telRL* recognition sequence



Supplementary Table S1. Restriction endonuclease information for downstream processing (removal) of dbDNA Vector backbone

RESTRICTION ENZYME	CUTSITE	NUMBER OF SITES
ApaLI	5′...G▼TGCAC...3′ 3′...CACGT▲G...5′	4
AseI	5′...AT▼TAAT...3 3′...TAAT▲TA...5′	4
BamHI-HF	5′...G▼GATCC...3 3′...CCTAG▲G...5′	4
ClaI	5′...AT▼CGAT...3 3′...TAGC▲TA...5′	3
FspI	5′...TGC▼GCA...3 3′...ACG▲CGT...5′	4
NdeI	5′...CA▼TATG...3 3′...GTAT▲AC...5′	3
PacI	5′...TTAAT▼TAA...3 3′...AAT▲TAATT...5′	3
RsrII	5′...CG▼GWCCG...3 3′...GCCWG▲GC...5′	4
SbfI-HF	5′...CCTGCA▼GG...3 3′...GG▲ACGTCC...5′	3
XbaI*	5′...T▼CTAGA...3 3′...AGATC▲T...5′	3

* Supplied with EnClose Cell-free dbDNA Synthesis Kit.

Revision History

REVISION #	DESCRIPTION	DATE
1.0	N/A	1/26

This product is intended for research purposes only. This product is not intended to be used for therapeutic or diagnostic purposes in humans or animals.

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