SinaClon TA Cloning Kit

(Sina-pNovel TA Lethal Vector)

NO Need To IPTG and XGal

For Research Use Only

Cat. No.: CL5841 **Storage: -20 ^{O}C**

Shipment: Wet Ice **Quantity: 20 Reactions**

1. **Repeating freezing-thawing reduces the efficiency of Sina-pNovel TA Lethal Vector.**
2. **Avoid vortexing T vector.**

Description: PCR TA Cloning kit is designed for rapid and efficient cloning of PCR products with 3'-dA overhangs. The kit use linearized Sina-pNovel TA Lethal Vector with 3'-dT overhangs to prevent vector recircularization, therefore resulting in high percentage of recombinant clones and low background.

Convenience – ready-to-use linearized 3'-dT overhang Sina-pNovel TA Lethal vector in this kit allows rapid clone selection by:

- M13 primer sites for PCR screening and sequencing.

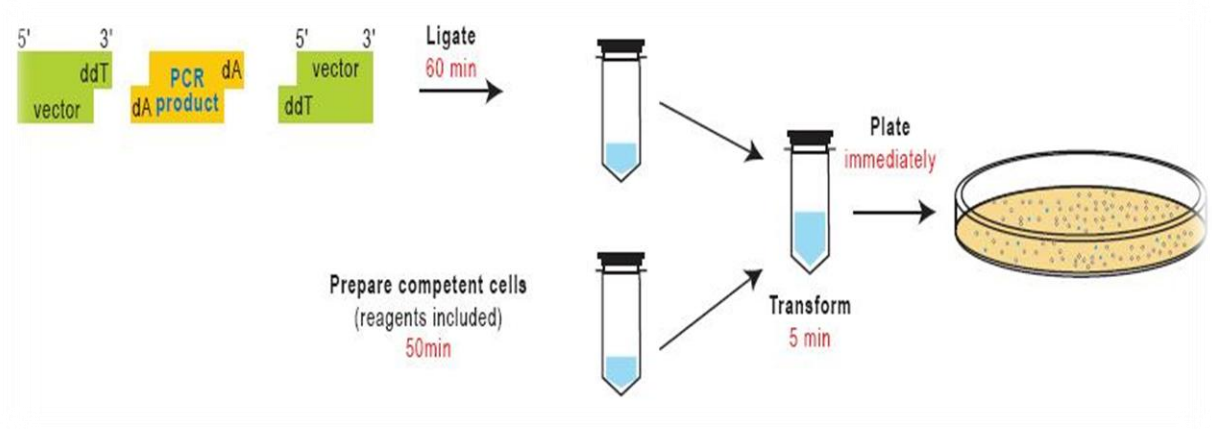


Figure1. PCR product cloning procedure

CLONING PRINCIPLE

Terminal transferase activity of certain thermophilic DNA polymerases, including *Thermus aquaticus* (*Taq*) polymerase adds a single adenosine to the 3'-ends of a double stranded DNA molecule so, most PCR fragments which amplified by non proofreading thermostable DNA polymerases like Taq polymerase possess single 3'-A overhangs.

The use of a linearized "T-vector" which has single 3'-T overhangs on both ends allows direct, high-efficiency cloning of PCR products, facilitated by complementarily between the PCR product 3'-A overhangs and vector 3'-T overhangs.
This method requires the use of T4 DNA ligase to covalently link the compatible ends of the DNA fragment and the linearized plasmid to forming a single cyclic molecule that is capable of autonomous replication in host component *E.coli*.

This procedure doesn't need enzymatic modification and primers designed with restriction sites for PCR reaction.
SinaClon TA cloning kit has five different stages:
1-PCR product generation by Taq DNA polymerase
2- PCR product ligation in the Sina-pNovel TA Lethal Vector
3- Transform of vector in to the component *E.coli*.
4- Colony selection method.
5- Identification of positive colonies

You may confirm presence and orientation of your cloned fragment by PCR, restriction or sequencing methods.

KIT CONTENTS:

1	Sina-pNovel TA Lethal vector (50ng/µl)	20 µl
2	T4 DNA Ligase (5 U/µl)	10 µl
3	10X Buffer Ligase	20 µl
4	Control insert (50-100 ng/µl)	10 µl
5	DNase Free Water	1000 µl

1- PCR product generation by *Taq* DNA polymerase

In general 10- 100ng of DNA is sufficient to use as a template for PCR. For PCR reaction you just need to add your own primers and DNA to master mix (Not provided).

The using of PCR Master Mix may help you to decrease unexpected bands or smears if not; you need to optimize your reaction. $MgCl_2$ concentrations and annealing temperature gradient may improve your reaction.
Otherwise you should cut single, sharp and discrete fragment after gel electrophoresis.
Avoid long exposure to UV light during gel excising. It dramatically damages your DNA fragment and decrease efficacy of cloning.
Always use fresh PCR products for cloning (<24h). Purification of PCR fragments by PCR purification kits or use of gel extraction kits always recommended before cloning procedure. Both procedures increase the number of recombinant colonies obviously.
If the template used in the PCR reaction contains the β-lactamase (Ampicillin resistance) gene, perform gel purification of the PCR band to reduce the number of background colonies.

Calculation the amount of insert for ligation:

Calculate the amount of insert for ligation based on the example below:
Example: For 500bp insert with 50ng of Sina-pNovel T/A Lethal Vector (2792bp)
2792bp/ 500bp= 5.58 (Vector is 5.58x larger than insert. You need 5.58x less insert)
For 50ng Sina-pNovel T/A Lethal Vector, 50ng/5.58= 8.96ng of insert for 1:1 ratio.
8.96ng x 3= 26.88ng or 26.88ng of insert for 1:3 ratio.

The ratio of 1:3 (vector: Insert) gives the best efficiency of ligation. You may wish to do a second ligation reaction at a ratio of 1:1 (vector: insert) if you are concerned about the accuracy of your DNA concentrations.
Do not use more than 2-3 µl of the PCR sample in the ligation reaction as salts in the PCR sample may inhibit the T4 DNA Ligase.
Add the following in a 0.2ml microcentrifuge tube:

Ligation Protocol:

Sina-pNovel TA Lethal Vector (50ng/µl)	1µl
Fresh and purified PCR product	µl*
10X Buffer Ligase	1µl
T4 DNA Ligase	0.5µl
Sterile Deionized Water	Up to 10µl
Total Volume	10µl

Incubate at 22°C for 3 hour. For maximum yield of ligation products, incubate the mixture at 16°C overnight.
Note: A control ligation reaction can be performed using 1µl of Control insert.
Proceed to Transforming Competent Cells.
You may store your ligation reaction at-20°C until you are ready for transformation.
Choosing suitable *E.coli* strains for competent cell preparation
E.coli cells are not included in this kit but E. coli strains should possess the following mutations which are important for transformation:
Some recommended *E. coli* strains for competent cell preparation are DH5α, TOP10, TOP10F' and XL1-Blue JM109 or other.
To transform competent strain, you should refer to the manufacturer's instructions.

Before Transformation:

LB agar plates with appropriate antibiotics (most commonly 100mg/ml of Ampicillin) should be available and warmed to room temperature.
A 42°C water bath or heater block should be available for the transformation.

Recommended Transformation Protocol:

1- Add 150ul of the overnight bacterial culture [1] to 1.5 ml of pre-warmed LB Broth. Incubate 60 min at 37°C in a shaker.
2- Collect cells by 5min centrifuge in 4000rpm, discard supernatant.
3- Resuspend cells in 300µl $cacl_2$ or $mgcl_2$.
4- Incubate on ice for 20minutes.
5- Collect cells by 1min centrifuge in 9000rpm, discard supernatant.[2]
6- Resuspend cells in 120µl $cacl_2$ or $mgcl_2$. Incubate on ice for 5 minutes. And repeat again stage 5.
7- In new tube add 5µl of the ligation reaction or 1 µl of supercoiled Control DNA (10-100pg). Incubate them on ice for 2minutes.
8- Add 100ul of the prepared competent cells to each tubes Containing DNA, mix gently by stirring with the pipette tip and incubate on ice for 30minutes.
9- Heat shocks the cells for 90 second in the 42°C water bath or heater block.
10- Immediately place the cells on ice for 10min.
11- Add 400µl of SOC or LB broth medium to the transformed cell vial.
12- Shake the cells at 37°C for 45-60 min at 225 rpm in a rotary shaking incubator.
13- Spread 100µl of the transformed cells on each labeled LB-Ampicillin agar plates.
Note: for more colonies, collect cells by 1min centrifuge, discard 400µl of supernatant and Resuspend cells in reminded supernatant and transformed cells on labeled LB-Ampicillin agar plates.

Note:

1. Overnight bacterial culture can obtain from inoculating of 2ml LB Broth with a single bacterial colony the day before transformation. Bacterial colony should not be older than 10 days.
2. Cells are susceptible to mechanical disruption, so treat them carefully.

Identification of Positive Colonies

After transformation, the positive colonies may be identified either by restriction mapping of the mini prepared plasmids or screening by PCR. PCR screening can be carried out with primers that flank the vector cloning sites or specific primers.
Analyze 4-6 white colonies for the presence and orientation of the DNA insert.
Gel electrophoresis analysis for the presence of the PCR product of the expected length.

Note. Due to considerable amount of recircularized vector plated on the surface of plate, colony PCR may give some false "negative results. Prior to clone analysis propagate short strikes of individual colonies on Ampicillin plates. Then use small amount of each for colony PCR.

The length of the amplified PCR product of a positive clone will be the size of the insert plus the bases flanking the two primers on the plasmid. This method is rapid, but may not work in some cases, especially when the insert is very large ≥3kb. Specific primers for the insert may also be used as PCR screening primers**.
**In this case annealing temperature depends to melt temperature of your primers.

Restriction analysis
To digest DNA from recombinant clones by restriction enzymes, plasmid should be extracted from an overnight bacterial culture using a convenient plasmid miniprep method. To speed up the process and to assure the quality of purified plasmid DNA, use the SinaClon Plasmid Extraction Kit (Mini Prep) Cat. No. EX6112).

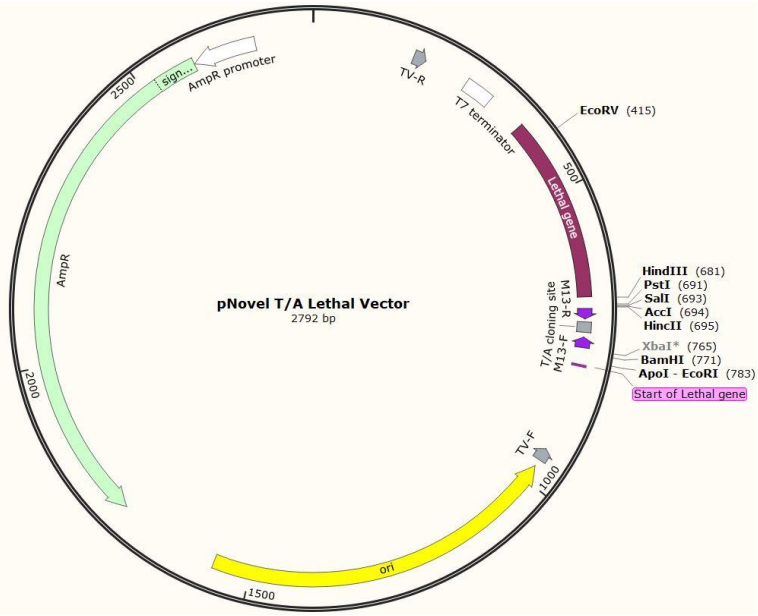
Sequencing
To assure the sequencing quality of purified plasmid DNA. Isolate plasmid DNA using a reliable plasmid miniprep method. Sequence the insert using standard sequencing primers with M13 primer.

M13F: 5'-GTAAAACGACGGCCAG-3'
M13R: 5'-CAGGAAACAGCTATGAC-3'

Screening by Restriction Mapping
For screening by restriction mapping, plasmids should mini prepared, then insert release by digestion with two unique restriction enzymes from the multiple cloning sites. Insert size is confirmed by agarose gel electrophoresis. The orientation of the insert in the plasmid can also be determined by appropriate restriction digestion, in some cases. If needed, the orientation and identity of the clone may be further confirmed by sequencing.

1- Randomly pick 4-6 white colonies and grow overnight at 37°C in 4ml of LB medium with 100µg/ml of Ampicillin.
2- Pellet 2-3 ml of the cells by centrifuging for 2 min. The remaining cells may be stored at 4°C for 1-2 days. For long term storage, add glycerol to 15%, and store at -70°C.
3- Isolate plasmids using Plasmid DNA purification system.
4- Digest ~50% of the total preparation with the appropriate restriction enzymes.
5- Run an agarose gel to determine which colonies have the correct insert size and/or orientation.

Genetic elements of Sina-pNovel TA Lethal Vector



Troubleshooting

Problem	Possible cause	Solution
Few or no colonies	Poor quality competent cells	Test transformation efficiency using supercoiled plasmid DNA (≥ 10^6 colonies per µg of supercoiled DNA is expected)
	The PCR product is blunt-ended	Perform PCR using *Taq* or polymerases which lack proofreading ability.
	Unsuccessful ligation	Perform a control ligation reaction using **Control insert**.
	The bacteria strain used did not possess *endA1* mutation.	Refer to "**Choosing suitable *E. coli* strains for competent cell preparation**".
White colonies do not have insert	Single 3'-dT overhangs on the vector degraded	Use another tube of vector. Avoid storing the vector for longer than one year or subjecting it to repeated freeze-thaw cycles.
A large colony surrounded by smaller white colonies	The smaller colonies are Ampicillin-sensitive satellite colonies.	Use fresh Ampicillin stock solution and LB/Amp plates.
		Do not pick the small colonies as they do not contain any plasmid.
False negatives in colony PCR	False-negatives in colony PCR.	Due to considerable amount of recircularized vector plated on the surface of the plate, colony PCR may give some false "negative results. Prior to clone analysis propagate short strikes of individual colonies on Ampicillin plates. Then use small amount of each for colony PCR.
Transformation efficiency is too low	Competent cells prepared from non-fresh bacterial culture.	Seed overnight culture from freshly streaked bacterial culture plate. Refresh bacterial strains weekly. For seeding of overnight DH5α® E.coli culture, use only one day old culture plates.
Sequence errors in the cloned insert	PCR product was damaged by UV light during excision from agarose gel.	Use a long wavelength UV (360 nm) light "box when excising DNA from the agarose gel. When a short "wavelength (254"312 nm) light "box is used, limit DNA exposure to UV to a few seconds. Keep the gel on a glass or on plastic plate during UV illumination. Alternatively, use dyes visible in ambient light to visualize DNA in standard agarose gels
	Errors in PCR primers.	If the cloned PCR product contains sequence errors or is missing 5' bases and the same error persists in more than one clone, reorder the PCR primers from a reliable supplier and repeat the procedure starting from the PCR step.