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Not intended for human diagnostic use

Zero Background Blunt Topoisomerase Cloning Kit

-for blunt end DNA fragment cloning

Rev. A. May. 1 2013

Cat. No.

With No Restriction Sites (Please refer to Appendix I):

C5851-25	Marker: Amp^R	Size: 25 reactions
C5851-50	Amp^R	50 reactions
C5852-25	Kan^R	25 reactions
C5852-50	Kan^R	50 reactions

With Multiple Restriction Sites (Please refer to Appendix II):

C5861-25	Marker: Amp^R	Size: 25 reactions
C5861-50	Amp^R	50 reactions
C5862-25	Kan^R	25 reactions
C5862-50	Kan^R	50 reactions

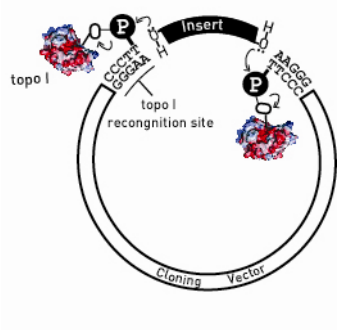
Store at -20°C

Note: Read and follow the instruction carefully to ensure optimal performance.

Description:

The Zero Background Blunt Topoisomerase Cloning Kit is designed for fast cloning of blunt ended DNA fragments up to 10 kb generated by high fidelity DNA polymerase such as KOD, Pfu and Phusion ext. DNA fragments obtained by restriction digestion or mechanical shearing can also be cloned after end polishing to become blunt ended. It utilizes DNA strand transfer activity of Viccinia virus topoisomerase I, Vaccinia virus DNA topoisomerase I forms a 3'-phosphoryl intermediate with the plasmid vector containing cleavage recognition motif of 5' CCCTT↓. Covalently bound topoisomerase I then transfer the incised vector DNA strand to the DNA fragment to be cloned with free 5'-OH terminuses (refer to the illustration below). This transferring reaction is rapid and reproducible. The pCloneEZ series cloning vectors included in this kit are high copy number plasmid engineered to tolerate mild toxic genes. Regions flanking the cloning site of pCloneEZ-NRS vectors do not contain any common restriction sites while regions flanking pCloneEZ have multiple common restriction sites for release of the cloned fragment by single or double restriction digestion. For fast cloning of extremely toxic genes please chose our advanced CloneRanger Topoisomerase Kit.

Fig.1 Strand transfer catalyzed by viccinia virus topoisomerase I



Components:

	Kit Size	
Components	**25 Rxns**	**50 Rxns**
pCloneEZ cloning vector, (20 ng/μl)	25 μl	50 μl
10X Enhancer	30 μl	60 μl
Control Insert lacZα (20ng/μl)	10 μl	10 μl
Blank Control Vector (100ng/μl)	20 μl	20 μl
M13F Sequencing Primer (10pmol)	100 μl	100 μl
M13R Sequencing Primer (10pmol)	100 μl	100 μl

Protocol:

Brief Summary (for first time user please refer to the detailed protocol below)

Mix: Vector + Fragment + Enhancer+ H2O = 10 ul

Standard Protocol (Total time needed: 40 – 100 minutes)	Fast Protocol (Total time needed: 5 – 65 minutes)
1. Incubate 5 min at RT	1. No incubation is needed
2. Transfer 5 μl to competent cell	2. Transfer 5 μl to competent cell
3. 30 min on ice	3. 2 min on ice
4. Heat shock (42°C, 30 seconds)	4. Heat shock (42°C, 30 seconds)
5. 2 min on ice	5. 2 min on ice
6. For Kan^R only: +SOC/LB, incubate @ 37°C, 60 min. For Amp^R go to step 7 directly	6. For Kan^R only: +SOC/LB, incubate @ 37°C, 60 min. For Amp^R go to step 7 directly
7. Plate	7. Plate
8. Incubate @ 37°C O.N.	8. Incubate @ 37°C O.N.

Detailed Protocol

⚠ First time users should carefully read through this protocol before starting the experiment.

1. Set up the topoisomerase cloning reaction by mixing the reagents in the order shown

Reagent	**Volume**
DNA Fragment	0.5–8 µL
pCloneEZ cloning vector	1 µL
10X Enhancer	1 µL
diH2O	X µL
Final Volume	**10 µL**

DNA fragments MUST NOT be 5' phosphorylated. Blunt ended DNA fragment is preferred. Highest cloning efficiency was observed from PCR fragments generated by high fidelity enzymes such as KOD, Phusion, Pfu etc. 20-200 ng of DNA fragments ranging from 100 bp to 5000 bp has been tested to give satisfactory results. Excess amount of DNA inserts e.g. ›300ng, will reduce cloning efficiency. Refer to the following table.

Table 1. Suggested optimal amount of fragment with various sizes:

Fragment size (bp)	Optimal amount (ng)
100-1000	20-50
1000-2000	50-100
2000-5000	100-200

A control reaction using 1 µL lacZα fragment as insert can be included to evaluate the cloning efficiency. The control reaction should generate better than 98% blue colonies when transformed into DH5 or DH 10B and plated on X-gal and IPTG containing plates.

* Store all reagents at –20°C when finished.

2. Mix the reaction gently and incubate for **5 minutes** at temperatures between 22–30°C. Or go to step #3 directly without incubation.

Recent R&D data indicate incubation at this step produce no detectable benefits as evident in the following data. In any case, do not let the incubation go beyond 5 minutes. Extended incubation for larger inserts up to 5 kb is unnecessary and may introduce background.

Table 2. Effects of incubation of reaction mixture vs. no incubation and instant transformation (with 1 hour recovery):

Fragment Size (bp)	Total Number of Transformants	
	Incubate 5 min, 25C	No Incubation
600	› 3000 colonies	› 3000 colonies
2000	ca. 300	ca. 500
3000	ca. 250	ca. 400
5000	ca. 100	ca. 100

3. Add 5 µL of the cloning reaction into 50 µL chemically competent E. coli and mix gently. Do not mix by pipetting up and down.

If not used for transformation immediately, reaction mixture can be stored at -20°C until use. Prolonged storage of the reaction mixture is NOT recommended.

4. Incubate on ice for 2–30 minutes.

Longer incubation on ice affects overall transformation efficiency to limited extend. The length of the incubation is at the user's discretion.

Table 3. Effects of incubation on ice on the total number of resulting transformants (with 1 hour recovery):

Fragment Size (bp)	Total Number of Transformants	
	30 min. Incubation	2 min. Incubation
600	›4000 colonies	›3000 colonies
2000	1346	913
3000	610	540
5000	289	241

5. Heat-shock the cells for 30 seconds at 42°C without shaking.

6. Immediately transfer the tubes to ice and incubate for 2 minutes.

7. If pCloneEZ with Amp resistance is used, immediately spread all from each transformation on a prewarmed plate containing ampicillin and incubate overnight at 37°C. ⚠ If pCloneEZ with Kan resistance marker is used, a mandatory incubation with LB, SOC or 2xYT medium for 60 minutes at 37°C has to be performed before plating onto pre-warmed kanamycin containing LB plates an incubate at 37°C overnight.

For fragments larger than 2kb, 1 hr recovery in rich media, even if pCloneEZ with Amp resistance marker is used, will dramatically increase the number of transformants. It is to the user's discretion whether recovery is needed based on individual purpose.

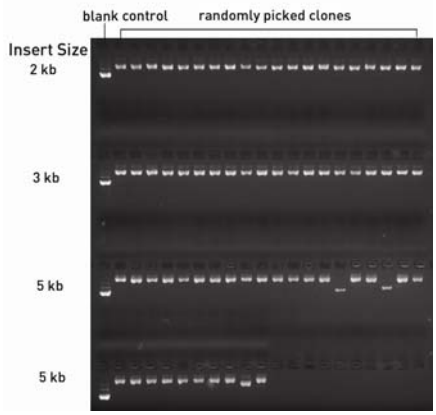
Table 4. Effects of 1 hour recovery after heat shock on total number of transformants:

Fragment Size (bp)	Total Number of Transformants	
	w/ 1 hr recovery in 2xYT medium	w/o recovery
600	›3000 colonies	›1000 colonies
2000	778	133
3000	627	68
5000	242	18

8. An efficient topoisomerase cloning reaction will produce hundred colonies. Pick ~10 colonies for analysis.

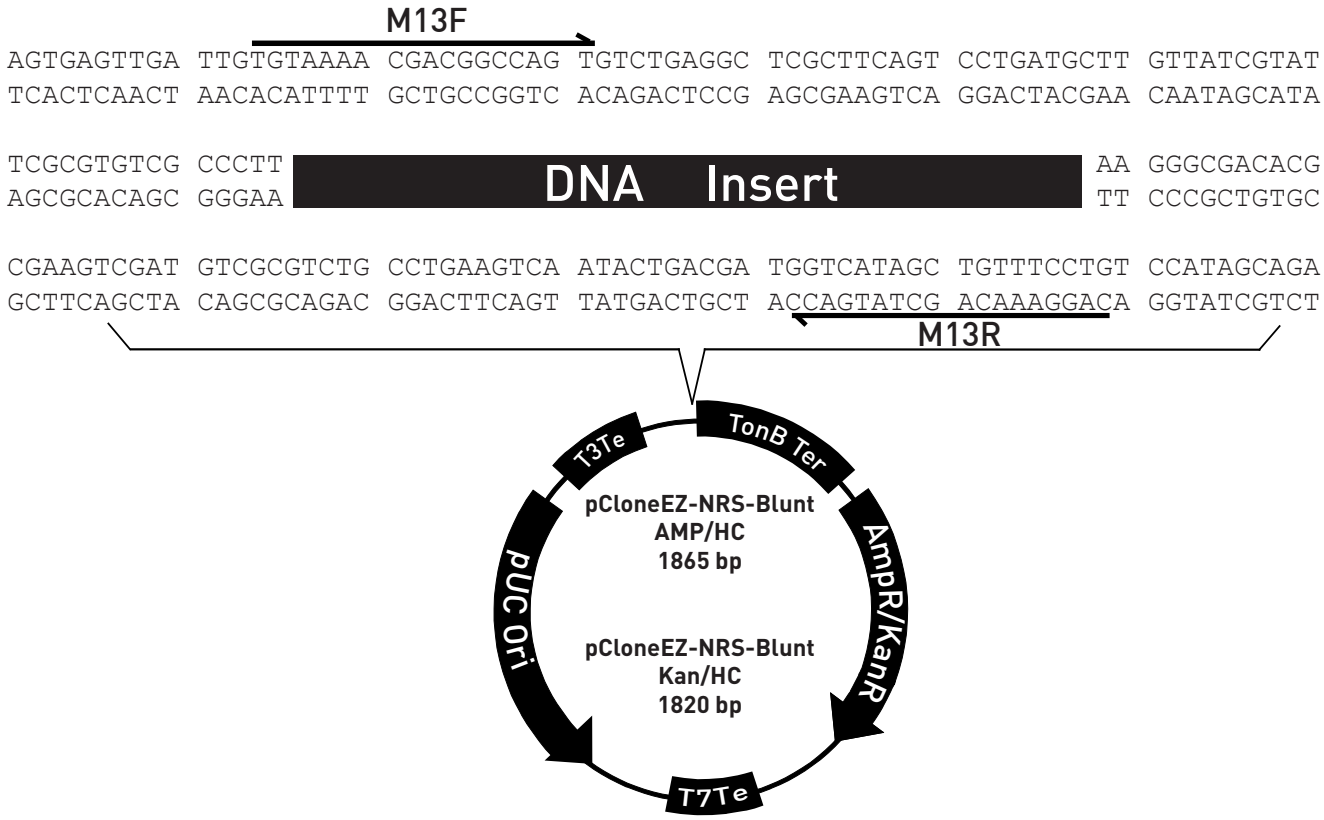
9. The included blank control vector is the recircularized pCloneEZ-Blunt vector without any insert. Therefore, it can be used as a size control during gel electrophoresis to identify recombinant plasmids containing inserts of interest.

Typical cloning results of PCR fragments with various sizes:



Appendix I: Vector information

pCloneEZ-NRS-Blunt-Amp/HC and pCloneEZ-NRS-Blunt-Kan/HC are high copy number plasmids for rapid cloning of blunt ended DNA fragments generated by proof reading DNA polymerases such as Pfu, KOD and Phusion etc. or end polished DNA fragments from restriction digestion or mechanical shearing. Both vectors tolerate mild toxic genes. The pCloneEZ-NRS vectors do not contain common restriction sites at their multiple cloning regions; this eliminates possible redundancy of restriction sites.



pCloneEZ-NRS-Blunt-Amp/HC Features:

TonB terminator:	167-198 bp
Beta-lactamase (AmpR):	302-1162 bp
T7Te terminator:	1186-1213 bp
pUC replication origin:	1225-1812 bp
T3Te terminator:	1834-1863 bp
M13F sequencing primer:	13-29 bp
M13R sequencing primer:	138-154 bp

pCloneEZ-NRS-Blunt Kan/HC Features:

TonB terminator:	167-198 bp
KanR:	302-1117 bp
T7Te terminator:	1141-1168 bp
pUC replication origin:	1180-1767 bp
T3Te terminator:	1789-1818 bp
M13F sequencing primer:	13-29 bp
M13R sequencing primer:	138-154 bp

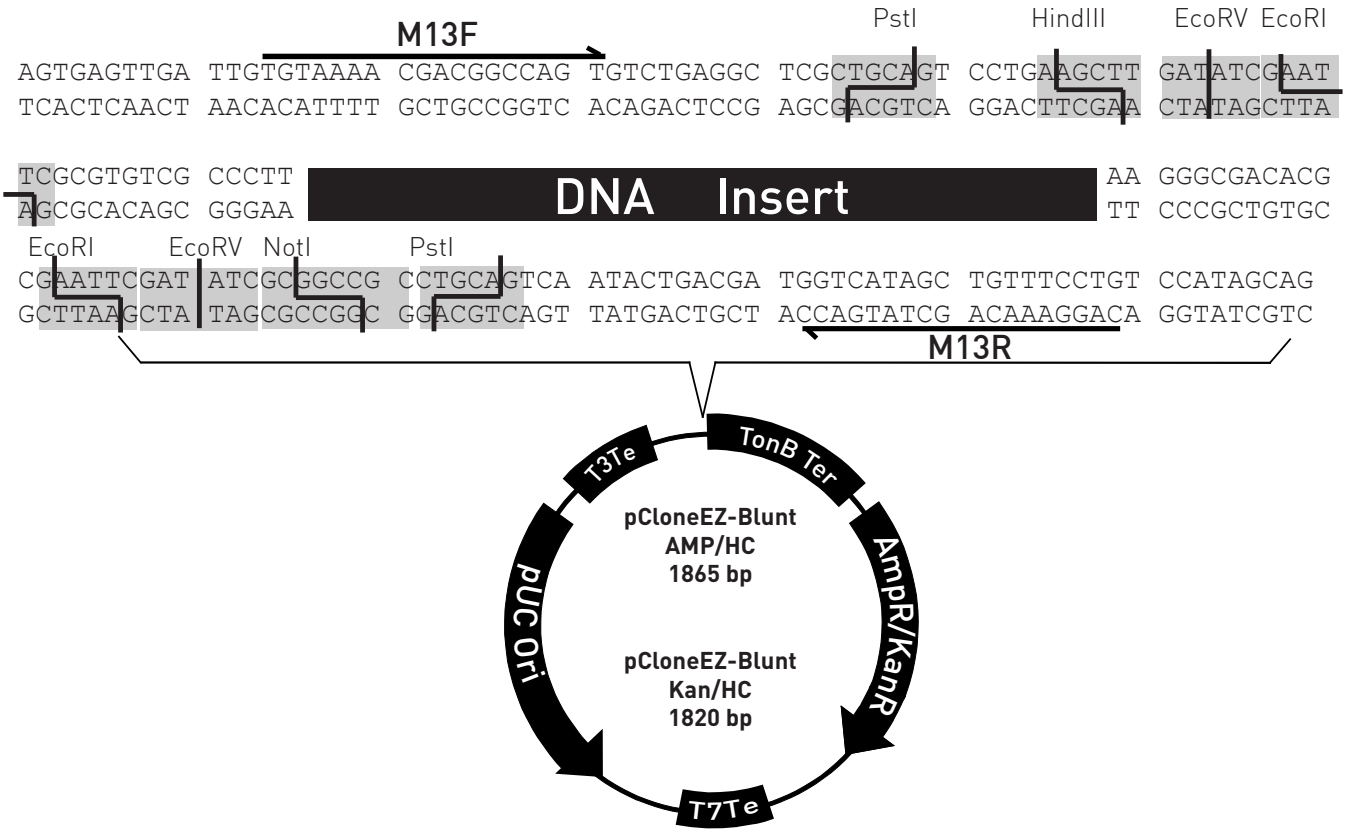
Sequencing Primers

M13F:
TGTAAAACGACGGCCAGT

M13R:
CAGGAAACAGCTATGACC

Appendix II: Vector information

pCloneEZ-Blunt-Amp/HC and pCloneEZ-Blunt-Kan/HC are high copy number plasmids for rapid cloning of blunt ended DNA fragments generated by proof reading DNA polymerases such as Pfu, KOD and Phusion etc. or end polished DNA fragments from restriction digestion or mechanical shearing. Both vectors tolerate mild toxic genes.



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M13F sequencing primer:	13-29 bp
M13R sequencing primer:	138-154 bp

pCloneEZ-Blunt Kan/HC Features:

TonB terminator:	167-198 bp
KanR:	302-1117 bp
T7Te terminator:	1141-1168 bp
pUC replication origin:	1180-1767 bp
T3Te terminator:	1789-1818 bp
M13F sequencing primer:	13-29 bp
M13R sequencing primer:	138-154 bp

Sequencing Primers

M13F:
TGTAAAACGACGGCCAGT

M13R:
CAGGAAACAGCTATGACC