Yeastern Biotech Co., Ltd

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T&A™ Cloning Vector Kit Protocol

Cat. No.
FYC001-20P
FYC002-20P

Manual Index

1. Vector map of T&A™ Cloning Vector

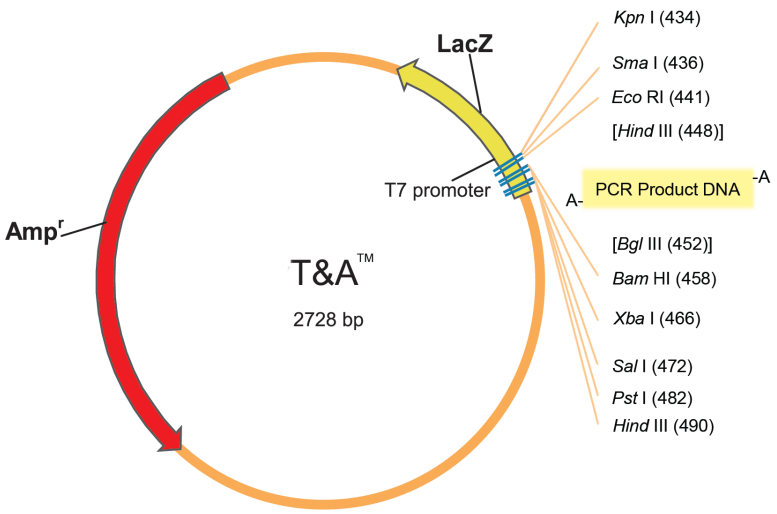

Figure1. Map and sequence reference points of T&A™ Cloning Vector

* Before the insert is incorporated into T&A™ Cloning Vector, there is only one *Hind*III site and no *Bgl*II site present in the multiple cloning region. After the incorporation, the 3'-A overhangs at both end of the insert will complement with the 3' overhanging T at the terminals of the vector and generate one additional *Hind*III site and one new *Bgl*II site. This merit of T&A™ vector makes cloning more economical and convenient.

Multiple cloning region	434 to 490
*Lac*Z start codon	511
*Lac*Z operator	531 to 548
*Lac*Z gene	511 to 149
Amp^r gene	2528 to 1671
T7 promoter	402 to 439
M13 forward primer	359 to 375
M13 reverse primer	507 to 528
β-lactamase coding region	1524 to 2528
Lac operon sequences	239 to 398, 488 to 725

2. DNA sequence of multiple cloning region in T&A™ Cloning Vector

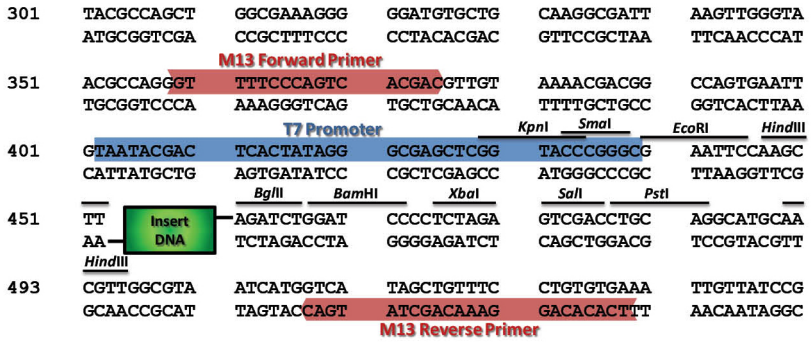

Figure 2. Multiple cloning site sequences of T&A™ Cloning Vector

3. Protocol for ligation using T&A™ Cloning Vector

1. Centrifuge T&A™ Cloning Vector and PCR DNA samples briefly to collect contents at the bottom of the tubes.
2. Vortex the ligation buffers vigorously before use.
3. Set up the following items as described below :

	Standard Control	Positive Control
Ligation Buffer A	1 μl	1 μl
Ligation Buffer B	1 μl	1 μl
T&A™ cloning vector	2 μl	2 μl
PCR product	X μl	****
yT_4 DNA ligase	1 μl	1 μl
Control DNA	****	3 μl
Add deionized water to a final volume of 10 μl		

4. Mix the reactions by pipetting.
5. Incubate the reactions for 5 to 15 min at 22°C. Alternatively, if higher transformation efficency is needed, incubate the reactions overnight at 4°C.
6. Transformation with appropriate competent cells accroding to user's downstream applications.

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4. Protocol for colony PCR

1. Prepare 25 µl of PCR reaction buffer in a microfuge tube as described below. Pick a single colony with a sterile toothpick and directly swirl it into PCR reaction buffer as the template.

PCR premixed buffer	
(O'in1 DNA polymerase premix, FYT201-100P)	23 µl
M13-F (10 µM)	1 µl
M13-R (10 µM)	1 µl

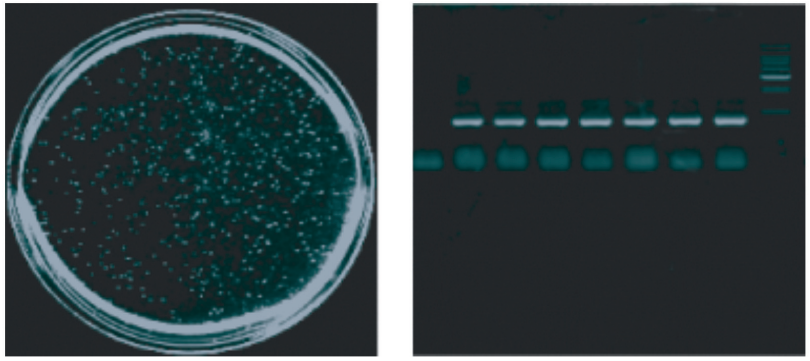
2. Set up PCR program

94°C	94°C	55°C	72°C	72°C	4°C
5min →	30sec →	30sec →	30sec[+++] →	7min →	∞

For 25 Cycles

[+++]The time duriation at 72°C is set according to the length of insert DNA.
(In general, DNA polymerase can synthesize 1 Kb of DNA in 1 minute.)

3. Run on 1% agarose gel
   Example : Using the control DNA provided in the T&A™ cloning vector kit as the insert DNA. The colony PCR result is shown as below.



*According to different primer designs, the size of colony PCR product seen on the agarose gel will change.
In this colony PCR, the size of the PCR product on the gel is larger than the insert DNA size by about 150 bp.

5. Product components

T&A™ Cloning Kit	Cat. No. FYC001-20P	
Components	Concentration	Volume
T&A™ Cloning Vector (20 reactions)	25 ng/µl	40 µl
Control Insert DNA	10 ng/µl	10 µl
yT_4 DNA Ligase	2 U/µl	20 µl
10x Ligation Buffer A	-----	50 µl
10x Ligation Buffer B	-----	50 µl
Forward Primer (M13-F)	10 µM	50 µl
Reverse Primer (M13-R)	10 µM	50 µl
Storage Condition: -20°C		

T&A™ Cloning Vector	Cat. No. FYC002-20P	
Components	Concentration	Volume
T&A™ Cloning Vector (20 reactions)	25 ng/µl	40 µl
Control Insert DNA	10 ng/µl	10 µl
Storage Condition: -20°C		

Suggestions

1. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by preparing single-use aliquots of the ligation buffers.
2. *Pfu* DNA polymerase possesses proofreading activity; it does not have the terminal transferase-like activity like Taq DNA polymerase. Ligation reactions using *Pfu* amplified DNA containing no A-tails will result in no positive colonies.
3. Methods for increasing the ligation efficiency :
   A. A-tailing: purified PCR product X µl
   10X PCR buffer 10 µl
   10 mM dATP 2 µl
   Taq 1 µl

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i. Add deionized water to a final volume of 100 µl.
ii. Incubate at 72°C for 1 hr.
Purify the A-tailed DNA and use in the ligation reaction.

B. If the maximum of transformants is required, incubate the reaction overnight at 4°C.

C. The optimial efficiency can be achieved by using a 1:3 molar ratio of vector DNA to the insert DNA.

D. Use competent cells with higher efficiency such as ECOS™ (>10^8cfu/µg DNA) series for transformation.

6. Restriction enzyme sites of T&A™ Cloning Vector

Enzyme	Position	Enzyme	Position	Enzyme	Position	Enzyme	Position	Enzyme	Position
*Aat*II	2664	*Asp*EI	1742	*Cfr*10I	1822	*Mam*I	457	*Ssp*I	2546
*Acc*65I	430	*Ava*I	434	*Dra*II	2718	*Nar*I	237	*Xba*I	466
*Acc*I	473	*Ban*II	428	*Eam*1105I	1742	*Nde*I	185	*Xma*I	434
*Acs*I	441	*Bam*HI	458	*Ecl*136II	426	*Pst*I	482	*Xmn*I	2341
*Afl*III	849	*Bcg*I	2281	*Eco*0109I	2718	*Sac*I	428		
*Ahd*I	1742	*Bpm*I	1812	*Eco*RI	441	*Sal*I	472		
*Alw*NI	1265	*Bsa*BI	457	*Hin*cII	474	*Sap*I	733		
*Apo*I	441	*Bsa*I	1803	*Hin*dII	474	*Sca*I	2222		
*Asp*700	2341	*Bsp*MI	485	*Kas*I	236	*Sma*I	436		
*Asp*718	430	*Bsr*FI	1822	*Kpn*I	434	*Sph*I	488		

Restriction enzymes that DO NOT cut T&A™ Cloning Vector

*Aat*I	*Bbs*I	*Bsi*WI	*Bst*BI	*Eco*47III	*Mro*I	*Nsi*I	*Ppu*10I	*Spe*I
*Acc*III	*Bcl*I	*Bsm*FI	*Bst*EII	*Eco*NI	*Msc*I	*Nsp*V	*Ppu*MI	*Ssp*BI
*Afl*II	*Bfr*I	*Bsm*I	*Bsu*36I	*Eco*RV	*Mun*I	*Pac*I	*Rsr*II	*Sty*I
*Age*I	*Bln*I	*Bsp*120I	*Cel*II	*Esp*I	*Nae*I	*PaeR*7I	*Sac*II	*Swa*I
*Apa*I	*Bpu*1102I	*Bsp*DI	*Cla*I	*Hpa*I	*Nco*I	*Pfl*MI	*Sex*AI	*Tth*111I
*Asp*I	*Bpu*AI	*Bsp*EI	*Csp*45I	*Ksp*I	*Ngo*MI	*Pin*AI	*Sfi*I	*Van*91I
*Asu*II	*Bsa*AI	*Bsr*GI	*Dra*III	*Mfe*I	*Nhe*I	*Pma*CI	*Sfu*I	*Xcm*I
*Avr*II	*Bse*AI	*Bss*HII	*Eag*I	*Mlu*I	*Not*I	*Pme*I	*Sgr*AI	*Xho*I
*Bbr*PI	*Bsg*I	*Bst*1107I	*Ecl*XI	*Mlu*NI	*Nru*I	*Pml*I	*Sna*BI	*Xma*III

Restriction enzymes that cut T&A™ Cloning Vector more than 2 times

Name	Position	Name	Position	Name	Position
*Aci*I	34sites	*Cfo*I	17 sites	*Mbo*I	16 sites
*Alu*I	17sites	*Csp*6I	169, 431,2221	*Mbo*II	7 sites
*Alw*44I	178,1163,2409	*Dde*I	6 sites	*Mcr*I	5 sites
*Alw*I	10 sites	*Dpn*I	16 sites	*Mnl*I	13 sites
*Aos*I	259,1964	*Dpn*II	16 sites	*Mse*I	13 sites
*Apa*LI	178,1163,2409	*Dra*I	1608,1627,2319	*Msl*I	1994,2153,2512
*Ase*I	620,679,1914	*Drd*I	98,957	*Msp*A1I	6 sites
*Asn*I	620,679,1914	*Dsa*V	12 sites	*Msp*I	13 sites
*Asp*HI	5 sites	*Eae*I	389,688,2130	*Mva*I	5 sites
*Ava*II	1880,2102	*Ear*I	297,733,2537	*Mvn*I	10 sites
*Avi*II	259,1964	*Eco*57I	1397,2409	*Mwo*I	13 sites
		*Eco*RII	5 sites	*Nci*I	7 sites
*Ban*I	4 sites	*Esp*3I	4, 46	*Nde*II	16 sites
*Bbv*I	12 sites	*Fnu*4HI	19 sites	*Nla*III	11 sites
*Bfa*I	4 sites	*Fnu*DII	10 sites	*Nla*IV	11 sites
*Bgl*I	252,1862	*Fok*I	5 sites	*Nsp*I	42,488,853
*Bmy*I	5 sites	*Fsp*I	259,1964	*Ple*I	5 sites
*Bsa*HI	237,2279,2661	*Hae*II	240,727,1097	*Psp*1406I	1968,2341
*Bsa*JI	4 sites	*Hae*III	11 sites	*Pvu*I	280,2112
*Bsa*WI	1055,1202,2033	*Hga*I	4 sites	*Pvu*II	309,673
*Bsi*EI	5 sites	*Hgi*AI	5 sites	*Rca*I	1569,2577,2682
*Bsi*HKAI	5 sites	*Hha*I	17 sites	*Rsa*I	170,432,2222
*Bsi*YI	6 sites	*Hin*dIII	(448),490	*Sau*3AI	16 sites
*Bsl*I	6 sites	*Hin*fI	7 sites	*Sau*96I	6 sites
*Bsm*AI	4 sites	*Hin*P1I	17 sites	*Scr*FI	12 sites
*Bsp*1286I	5 sites	*Hpa*II	13 sites	*Sfa*NI	8 sites
*Bsp*HI	1569,2577,2682	*Hph*I	7 sites	*Sfc*I	5 sites
*Bsp*WI	13 sites	*HPY*188I	10 sites	*Sno*I	178,1163,2409
*Bsr*BI	541,782,2583	*Ita*I	19 sites	*Taq*I	473,949,2393
*Bsr*DI	1803,1977	*Ksp*632I	297,733,2537	*Tfi*I	684,824
*Bsr*I	11 sites	*Mae*I	4 sites	*Tha*I	10 sites
*Bst*NI	5 sites	*Mae*II	5 sites	*Tru*9I	13 sites
*Bst*UI	10 sites	*Mae*III	11 sites	*Tsp*509I	8 sites
*Bst*YI	7 sites			*Xho*II	7 sites

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7. Suggestions and notes

1. For blue-white selection, plating should be done onto agar plates containing 20-50 μg/ml ampicillin plus 0.1-0.5 mM IPTG and 40-60 μg/ml X-gal. Alternatively, the IPTG and X-gal can be spreaded evenly onto a 20-50 μg/ml ampicillin plate at least 2 hr prior to plating. After the colonies grow for at least 14 hr, the plates can be put at 4°C and the blue color will continue to develop.

2. When coupled with the colony PCR technique, clones can be screened easily and precisely.

3. Multiple freeze-thawing DOES NOT affect the quality of T&A™ Cloning Vector, but exposure to frequent temperature changes will degrade ATP resulting in poor ligation.

4. For questions not addressed here, please visit our web site for details. http://www.yeastern.com

8. Troubleshooting

Symptom	Comments	Suggestions
No colonies	Problems in transformation or use low-efficiency competent cells.	Self-ligated vector should only yield < 50 colonies, while with A-tailed insert DNA contol the transformation should yield > 500 colonies. If there is no colony on the plate, use high-efficiency competent cells to overcome the problem.
Less than 100 transformed colonies grow on selection plate. When checking the colonies with colony PCR using M13F&R primers, false inserts of 0.15 and 1.8 kb fragments are obtained.	1. Improper molar ratio of the vector DNA to the insert DNA. 2. Bad A-tailed insert DNA. 3. The 0.15kb fragment is derived from reaction of self-ligation of the vector itself. The 1.8 kb fragment is derived from the residue in production process.	1. Check the A-tailed insert DNA quality. 2. Using a 1:3 to 1:6 molar ratio of the vector DNA to the insert DNA. 3. Do not try to amplify and sequence the clones when less than 100 transformed colonies obtained. Most of them contain false inserts. 4. If the size of your insert DNA is about 1.6~1.8 kb, please perform colony PCR by specific primers to check the clone at first. If the clone is correct, then sequence it with M13 primers.
High colony number, but high percentages of blue colonies with insert DNA.	1. Improper ligation reaction. 2. DNA is inserted, but it's not disrupting the expression of LacZ gene or the insertion generates in-frame fusion of the coding region to lacZ gene.	1. PCR DNA should be gel-purified or spin column-cleaned. 2. Avoid multiple freeze-thaw and exposure to frequent temperature changes by preparing single-use aliquots of the ligase buffer. 3. If a maximum of transformants is required, incubate the ligation reaction overnight at 4 °C. 4. If multiple PCR products occur, target DNA should be gel-purified before ligation. 5. Use colony PCR to screen both of the blue or white colonies.
No blue colonies are present on the plate.	1. Ampicillin is inactive. 2. IPTG/X-Gal is insufficient or inactive.	1. Check if ampicillin plates are prepared properly used within 1 month. 2. Check if the IPTG/X-Gal are fresh and the IPTG/X-Gal plates should be stored in dark and cold place.

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