

TA Cloning Kit User's Instruction

Description

TA Cloning Kit is a convenient system for the cloning of PCR products. Both pKRX-T vector and T4 DNA Ligase are included in this kit. The pKRX-T vector was made by cutting the pKRX plasmid which was developed by cloning a cassette containing two *XcmI* recognition sites between *EcoRI* and *SalI* sites of pBluescript SK(+), after digestion by *XcmI*, generated single 3'thymidine overhangs on both ends of the vector.

These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of PCR products into the plasmids by preventing recircularization of the vectors and providing compatible overhangs for PCR products generated by certain thermostable polymerases such as *Taq* and *Tth* which often add a single deoxyadenosine to the 3'-ends of the amplified fragments.

pKRX-T Vector Structure

1. Sequence of pKRX-T Vector Promoters and MCS

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5' ... GTAAA ACGAC GGCCA GTGAG GCGGC GTAAT ACGAC TCACT ATAGG GCGAA
3' ... CATTI TGCTG CCGGT CACTC GCGGG CATTI TGCTC AGTGA TATCC CGCTT
      M13/pUC sequencing primer(-20)          T7 Promoter
  
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          Eco0109 I
      Acc65 I  Apa I      Hinc II
      Kpn I   Bsp120 I   Xho I   Sal I
          Xmi I
TTGGG TACCG GGCOC COCCT CGAGG TOGAC CAGAC GT 3'          AG
AAOCC ATGGC CCGGG GGGGA GCTOC AGCTG GTCTG C          3' TTC
                                     ↑
                                     ( cloned insert )
  
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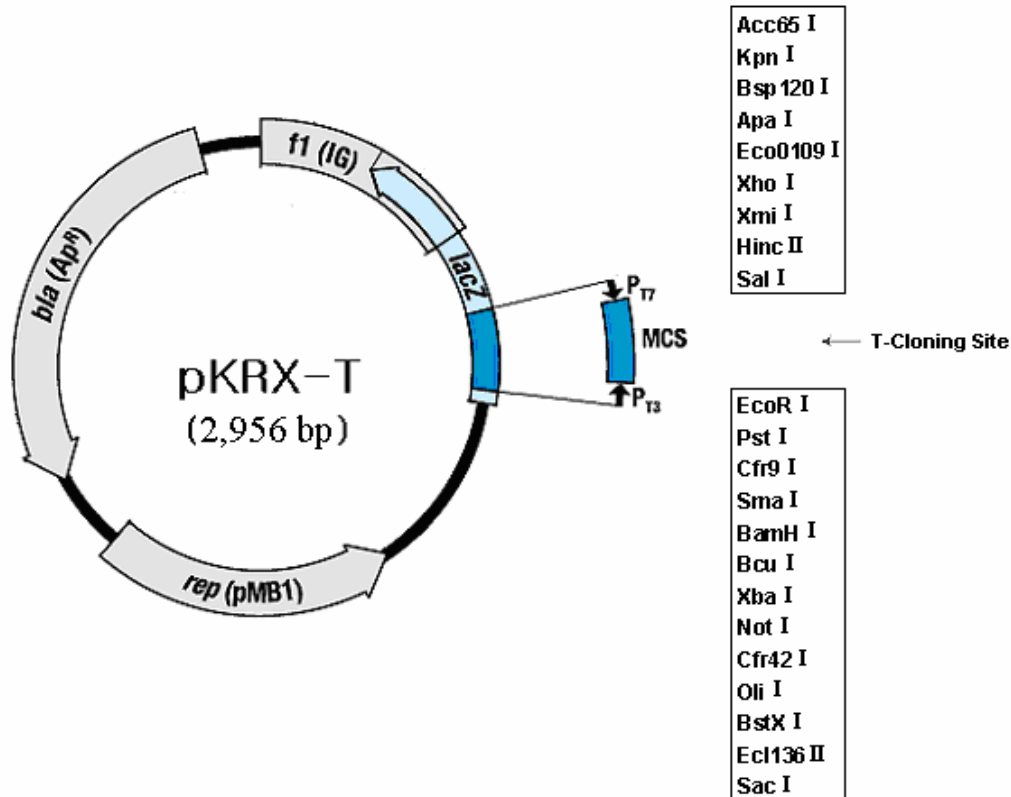
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          Cfr9 I
      EcoRI  Pst I   Sma I  BamHI   Bcu I   Xba I   Eco52 I
          Not I
GCTGG TGAAT TCCTG CAGCC CGGGG GATCC ACTAG TTCTA GAGCG GCCGC
CGACC ACTTA AGGAC GTCGG GCCOC CTAGG TGATC AAGAT CTCGC CGGCG
  
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      Cfr42 I
      BstX I  Ecl136 II
      Oli I   Sac I
CAOOG OGGTG GAGCT CCAGC TTTTG TTCCC TTTAG TGAGG GTTAA ... 3'
GTGGC GCCAC CTCGA GGTGC AAAAC AAGGG AAATC ACTCC CAATT ... 5'
                                     T3 Promoter
  
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pKRX-T Vector Map



Product Components

pKRX-T Vector (30ng/μl)	20μl × 1
Control Insert DNA (10ng/μl)	20μl × 1
T7 Promoter Primer	1nmol × 1
T3 Promoter Primer	1nmol × 1
T4 DNA Ligase (3U/μl)	15μl × 1
10×T4 DNA Ligase Buffer	100μl × 1

Storage -20°C

Quality Control

- 1 . When molar ratios of the Control Insert DNA (500bp) to the vectors is 1:1 to 50:1, > 80% of the whole transformants are recombinant colonies.
- 2 . After cloning the Control Insert DNA, sequencing to confirm MCS and thymidines.

Protocol

A . PCR Product Cloning

1 . PCR product Purification and Quantification

The PCR product to be ligated should be gel-purified and determine the amount of DNA by comparing with markers.

2 . Ligation

Set up ligation reactions as described below.

10×T4 DNALigase Buffer	1 μ l
pKRX-T (30ng/ μ l)	1 μ l
Gel-purified PCR Product	X μ l*
T4 DNA Ligase (3U/ μ l)	0.5 μ l
ddH ₂ O	up to 10 μ l

* The best molar ratios of the PCR product to the vector are 2:1~10:1. Mix the reactions by pipetting. Incubate the reactions 3 hour or overnight at 16°C. Longer incubation times will increase the number of transformants. (Generally; incubation overnight at 16°C will produce the maximum number of transformants) .

3 . Transformations

- a. Thaw 100 μ l competent cells on ice.
- b. Add 5 μ l ligation reaction, swirl tube, incubate on ice for 30 minutes.
- c. Heat-shock the cells for 90 seconds in water bath at exactly 42°C (Do not shake). Immediately return the tubes to ice for 2 minutes.
- d . Add 900 μ l room temperature LB medium to the tubes. Incubate for 1 hour at 37°C with shaking (~150rpm).
- e . Plate 100 μ l of each transformation culture onto duplicate LB/ampicillin plates or LB/ampicillin/IPTG/X-Gal plates. If a higher number of colonies are desired, the cells may be pelleted by centrifugation at 1,000 \times g for 10 minutes, resuspended in 200 μ l of SOC medium, and 100 μ l plated on each of two plates.
- f . Incubate the plates overnight (16~24 hours) at 37°C.

4 . Identification of recombinant colonies

Take cells from fresh plates (or take white cells from LB/ampicillin/IPTG/X-Gal plates), culture in LB/ampicillin medium overnight at 37°C, isolate plasmid. Identify the recombinant colonies by double digestion with two different restriction endonucleases

or PCR with T7/T3 promoter primers. Colony PCR Method see B . Control Insert DNA Cloning.

B . Control Insert DNA Cloning

1 . Ligation

Set up ligation reactions as described below.

10×T4 DNA Ligase Buffer	1 µl
pKRX-T (30ng/µl)	1 µl
Control Insert DNA (10ng/µl)	2~3 µl
T4 DNA Ligase (3U/µl)	0.5 µl
ddH ₂ O	up to10 µl

Mix the reactions by pipetting. Incubate the reactions 3 hour or overnight at 16°C. Longer incubation times will increase the number of transformants (Generally; incubation overnight at 16°C will produce the maximum number of transformants) .

2 . Transformations

- a . Thaw 100µl competent cells on ice.
- b . Add 5µl ligation reaction , swirl tube, incubate on ice for 30 minutes.
- c . Heat-shock the cells for 90 seconds in a water bath at exactly 42°C (Do not shake). Immediately return the tubes to ice for 2 minutes.
- d . Add 900µl room temperature LB medium to the tubes. Incubate for 1 hour at 37°C with shaking (~150rpm).
- e . Plate 100µl of each transformation culture onto duplicate LB/ampicillin plates or LB/ampicillin/IPTG/X-Gal plates. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1,000 × g for 10 minutes, resuspended in 200µl of SOC medium, and 100µl plated on each of two plates.
- f . Incubate the plates overnight (16~24 hours) at 37°C.

3 . Identification of Recombinant Colonies by Colony PCR Method

- a . Mixed reagents as described below into a 1.5 ml tube, divide this 250 µl mixture to 10 thin-wall tubes on ice. Every colony PCR reaction is 25µl. Adjust the amount of the mixture according to what you need.

10×Taq DNA Polymerase Buffer	25 µl
dNTPs(10mmol/L)	5 µl
T7 promoter primer (10µmol/L)	5 µl
T3 promoter primer (10µmol/L)	5 µl
Taq DNA Polymerase	10 Units
ddH ₂ O	up to 250 µl

b . Take cells from plates with sterile tips into 25µl mixtures.

c . Perform PCR cycles according to the following condition

94°C for 5 minutes; 94 °C for 30 seconds, 42 °C for 30 seconds, 72 °C for 50 seconds, 30cycles.

d . PCR products from recombinant colonies are 660 bps, but these from negative ones are 160 bps (between T7 Promoter and T3 Promoter).

General Considerations

- 1 . For 500bps insert DNA , > 80% of the whole transformants are recombinant colonies when molar ratios of insert DNA to the vectors are 1:1 to 100:1; but the most optimal molar ratios of insert DNA to the vectors are 2:1~10:1, at that time, > 90% are recombinant ones.
- 2 . Generally, the ratio of blue colonies is about 5~10% when LB/ampicillin/IPTG/X-Gal plates are used. Since the ratio of white colonies is > 90%, blue/white color selection is not needed.
- 3 . When a ligation reaction with 30ng of pKRX-T (but without insert DNA) is set up as a background control, the number of colonies from the background control is about 10% of that from Control Insert DNA Cloning or PCR product cloning group.
- 4 . The pKRX-T vector is from pBluescriptSK(+).
- 5 . The control Insert DNA is gel-purified PCR product (500bps).
- 6 . When Identifying the recombinant colonies by PCR with T7/T3 promoter primers, pay attention that there is 160 bases between T7 Promoter and T3 Promoter.
- 7 . Thaw pKRX-T vector and 10×T4 DNA Ligase Buffer at room temperature or 30°C.