



# Genaxxon BioScience T/A Cloning Kit

For fast cloning of DNA fragments with A overhangs

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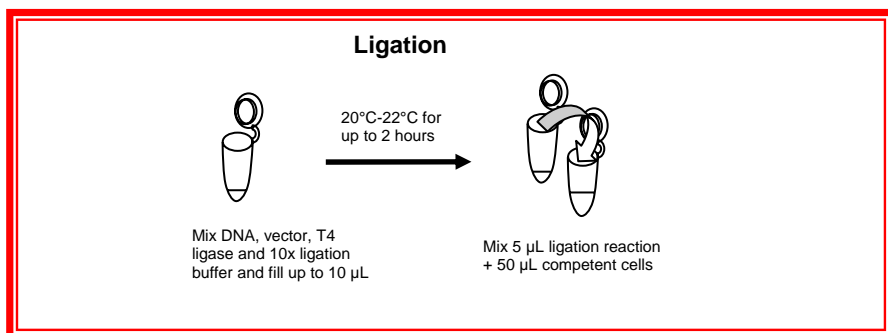
Version: 15102008

<b>Product</b>	<b>Cat#</b>	<b>Package size</b>
pMBL-T/A Cloning Kit	M3164.0020	20 reactions
pMBL-T/A Cloning Kit	M3164.0040	40 reactions

## Manual Contents

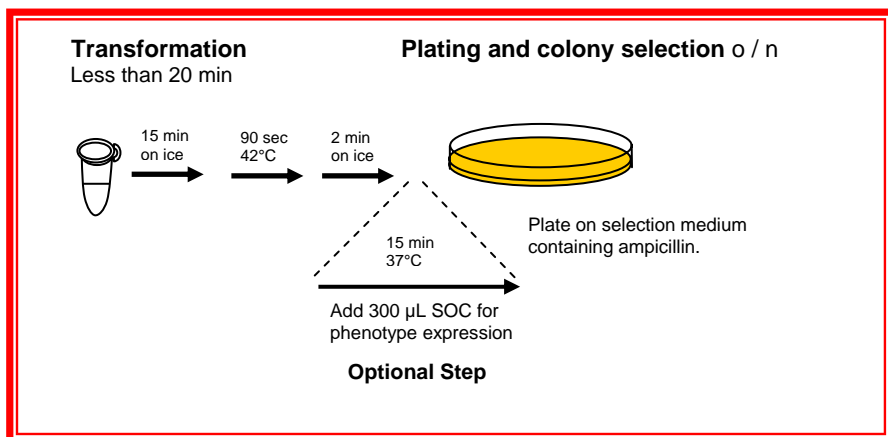
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## Protocol overview Cloning



## Protocol overview Transformation (not part of the kit)

e.g. with *E. coli* TZ101α chemically competent cells (M3434 or M3435)



## Kit Contents & Storage Information

Item	Amount	Storage
pMBL-T/A vector	1.0 µg (50 ng/µL) (20 reactions)	- 20°C
T4 DNA Ligase	100 Wunits (5 WU/µL) (20 reactions)	- 20°C
10X T4 DNA Ligase Buffer		- 20°C

## Abbreviations

L, mL, µL	Litres, milli litres, micro litres respectively
g, µg, ng	grams, micro grams, nano grams respectively
Wunits, WU	Weiss-units
bp	Base pairs
LB	Luria Bertani medium
XGal	5-Brom-4-Chlor-3-indolyl-β-D-galactopyranoside
TE	Tris-EDTA (10 mM Tris-HCl, pH8.1, 1 mM EDTA)
Amp	Ampicillin
RT	Room temperature (18°C – 24°C)

## Related products / overview

- Genaxxon DNA Gelextraction Kit S5344
- Genaxxon JustSpin Gel Extraction Columns S5337
- Genaxxon SpinClean Spin Columns S5304
- Genaxxon QuickClone Kit M3457
- Genaxxon *Taq* DNA Polymerase M3001
- Genaxxon Insert Inspector M3458
- Genaxxon chemically competent cells *E. coli* TZ101α M3434
- Genaxxon chemically competent cells *E. coli* TZ102α M3435
- Genaxxon Alligator M3430
- Genaxxon T4 DNA Ligase M3027

## Notes on Warranties and Disclaimer

Genaxxon is dedicated to your success and every batch of this product is tested with an extensive routine procedure to make sure that it meets all your needs. However, it has neither been developed nor tested for a specific application.

### This product is for research use only. For *in vitro* use only.

Genaxxon's liability with respect to any product is limited to the replacement of the product. No other warranties are provided by Genaxxon. Genaxxon is not liable to any direct, indirect, incidental or consequential damage arising out of or in connection with the use of any of Genaxxon products.

## Expected results

Depending on the quality and size of the DNA fragment, varying numbers of potentially positive white clones will grow, typically more than 50% of total transformants. They contain inserts of the fragment in clockwise or counterclockwise orientation.

Positive clones may be identified by isolation of the plasmid DNA followed by restriction analysis. For more convenient checking of your results, Genaxxon offers a colony screening kit:

- Genaxxon Insert Inspector (M3458)

## Remarks

Not all blue colonies are negative clones. The insert may be in frame with  $\beta$ -galactosidase alpha-peptide. In this case also positive blue colonies are obtained. As with cloning kits other suppliers this effect happens frequently with short inserts. In this case **more** blue colonies will be obtained **than white** colonies.

## Trouble shooting guide

### No white colonies

- Use more DNA-fragment (up to 300 ng).
- Make sure to purify the insert DNA.
- Some cloned inserts might interfere with cellular functions of *E. coli*. To recover such clones, try the incubation of the plates at RT or 28°C rather than at 37°C.
- Plate out more cells to make sure inserts of both orientations are recovered since sometimes one orientation of the insert works better than the other.
- Increase ligation time (up to 2 h).
- Sometimes small inserts may not disrupt the reading frame of  $\beta$ -galactosidase and therefore clones remain blue or light blue.

### White colonies do not contain (correct) inserts

- Since smaller fragments are inserted much easier than larger ones, make sure to purify the fragment by gel electrophoresis. Thus, short DNA molecules like primer dimers are removed.

### Only few colonies

- Make sure to keep competent cells on ice all time prior to heat shock.
- Do not increase ampicillin concentration above 100  $\mu$ g/L.
- Perform the phenotype expression step.
- Use a larger amount of DNA for transformation.

## Description

The pMBL-T/A Cloning Kit is a convenient system for the cloning of PCR products. The vector is prepared by cutting pMBL vector with *EcoRV* and adding a 3' terminal thymidine to both ends. These single 3'-T overhangs at the insertion site greatly improve the efficiency of ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases. These polymerases often add a single deoxyadenosine, in a template-independent fashion, to the 3'-ends of the amplified fragments.

The high copy number pMBL vector contains a T3 RNA polymerase promoter in the 3'-region of the Multiple Cloning Site (MCS) for in vitro RNA transcription. Insertional inactivation of the  $\alpha$ -peptide allows recombinant clones to be directly identified by color screening on indicator plates. The MCS of the vector includes the following unique restriction sites: *Apal*, *Sall*, *BamHI*, *SpeI*, *EcoRI*, *SmaI*, *StuI*, *EcoRV* (lost after digestion and T addition), *HindIII*, *SacI*, *NotI*, *NheI*, *BglII*, *XbaI*, *XhoI*, *MluI* and *PstI* and two *KpnI* sites flanking the MCS.

The pMBL-T/A Cloning Kit includes T4 DNA Ligase and Buffer for ligation in up to 2 hours. In our experience extended ligation times do not increase the number of colonies after transformation, but transformation should be done, albeit with a little lower efficiency, after 1 hour of ligation.

## Shipping and Storage

Overnight shipment at room temperature does not affect product performance but storage at -20°C is highly recommended for long term storage.

## Quality Control

More than 90% white colonies in a transformation with supplied control insert.

The total number of colonies in the supplied control insert should be higher than 1000, provided that *E. coli* cells have a competence of more than  $1 \times 10^7$  colonies/ $\mu$ g of circular pUC18.

## Recommended Protocol

1. Mix the following:

1 $\mu$ L of T4 DNA Ligase 10X Buffer	(1x final concentration)
1 $\mu$ L of pMBL-T/A vector	(5 ng/ $\mu$ L final concentration)
x $\mu$ L of DNA insert containing solution	
1 $\mu$ L of T4 DNA Ligase	(0.5 U/ $\mu$ L final concentration)
and add H <sub>2</sub> O up to 10 $\mu$ L	

We recommend a 1:5 molar ratio of vector to insert according to the following formula:

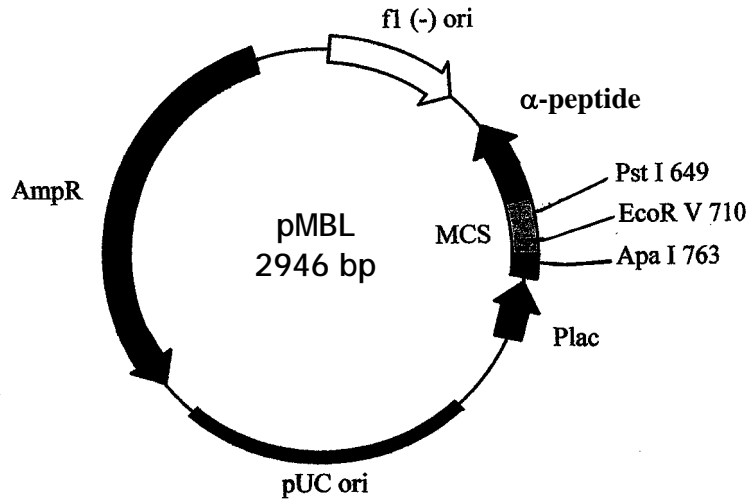
$$\text{ng insert} = \frac{\text{ng of vector} \times \text{insert length in bp}}{\text{vector length in bp} \times \text{ratio vector to insert}}$$

2. Incubate the above mixture between 20°C to 22°C for up to 2 hours.

3. Transform 5  $\mu$ L into 50  $\mu$ L of competent *E. coli* cells.

We recommend not to transform with a volume of ligation mix higher than 7.5  $\mu$ L.

## The vector pMBL

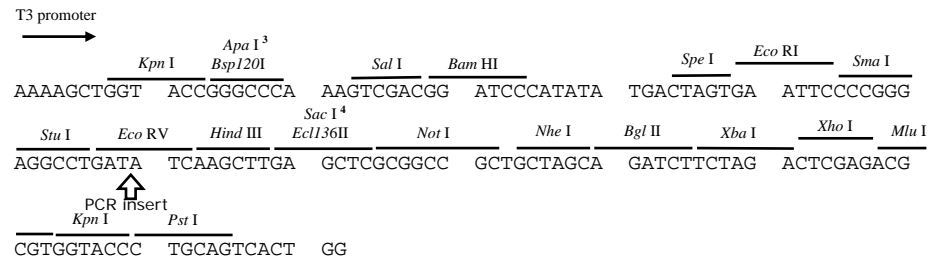


```

1741 tttttgtttg caagcagcag attacgcgca gaaaaaaagg atctcaagaa gatcctttga
1801 tctttttctac ggggtctgac gctcagtgga acgaaaaactc acgttaaggg attttgggtca
1861 tgagattatc aaaaaggatc ttcacctaga tccttttaaa ttaaaaatga agttttaaat
1921 caatctaaag tatatatgag taaacttggt ctgacagtta ccaatgctta atcagtgagg
1981 cacctatctc agcgatctgt ctatctcgtt catccatagt tgccctgactc cccgctcgtgt
2041 agataactac gatacgggag ggcttaccat ctggccccag tgmtgcaatg ataccgcgag
2101 acccaagctc accggctcca gatttatcag caataaacca gccagccgga agggccgagc
2161 gcagaagtgg tctctgcaact ttatccgcct ccatccagtc tattaatgtg tgccgggaag
2221 ctagagtaag tagttcgcca gttaatagtt tgcgcaacgt tgttgccatt gctacaggca
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2341 ggcgagttag atgatccccc atggttggtgca aaaaagcggg tagctccttc ggtcctccga
2401 tcggtgtcag aagtaagttg gccgcagtggt tatcactcat ggttatggca gcactgcata
2461 attctcttac tgtcatgcca tccgtaagat gcttttctgt gactgggtgag tactcaacca
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2641 ggcaaaaact ctcaaggatc ttaccgctgt tgagatccag ttogatgtaa cccactcgtg
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2761 gaaggcaaaa tgccgcaaaa aaggggaataa gggcgacacg gaaatgttga atactcatac
2821 tcttcctttt tcaatattat tgaagcattt atcaggggta ttgtctcatg agcggataca
2881 tatttgaatg tatttagaaa aataaacaaa taggggttcc gcgcacattt ccccgaaaag
2941 tgccac
  
```

## Map of vector pMBL

The locations of genes are indicated by arrows.  
Selected unique restriction endonucleases sites are shown.



## Additional Information

### Sequence of pMBL-T/A vector

21-327	f1(-) origin of replication
460-798	$\beta$ -galactosidase $\alpha$ -peptide
619-746	Multiple Cloning Site (MCS) (complement)
802-923	Lac promoter (complement)
1143-1810	pUC replication origin
1958-2818	$\beta$ -lactamase resistance cassette (AmpR) (complement)

```
1 ctgacgcgcc ctgtagcggc gcattaagcg cggcgggtgt ggtggttacg cgcagcgtga
61 ccgctacact tgccagcgcc cttagcggcc ctcctttcgc tttcttccct tcctttctcg
121 ccacgttctc cggctttccc cgtcaagctc taaatcgggg gctcccctta gggttccgat
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1381 tggcgctttc tcatagctca cgctgtaggt atctcagttc ggtgtaggtc gttcgtcca
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1681 tcggaaaaag agttggtagc tcttgatccg gcaaacaaac caccgctggt agcggtaggt
```

## Before you start: Helpful hints for optimum results

### DNA fragment: Make sure that you use an ample amount of high-quality, purified DNA

The outcome of the cloning procedure largely depends on the quality of the DNA fragment used. We recommend to perform size selection by purifying the DNA by agarose gel electrophoresis followed by extraction of the fragment using a suitable kit, e.g.

- Genaxxon JustSpin Gel Extraction Columns S5337.0050
- Genaxxon SpinClean Spin Columns S5304.0100

In the case of cloning a PCR fragment, the gel electrophoresis step may be omitted – but the PCR product should be purified using a suitable kit, e.g.

- Genaxxon PCR Clean-up Kit S5345.0050
- Genaxxon PSI Clone 96 HTS PCR purification Kit S5303.2196

Since many methods for clean-up of PCR fragments do not remove unspecific products like primer dimers, these small products could also be integrated in the vector pMBL during the cloning step and may produce false positive (white) clones with small inserts.

Make sure to use sufficient amounts of DNA (> 50 ng; if the size of the fragments is large, use more). The smaller the fragment, the easier it is inserted.

### Buffers: Keep buffers on ice. Use buffer aliquots and avoid numerous freeze-thaw cycles.

To the best of our knowledge, both buffers are stable over many freeze-thaw cycles if the solutions are kept on ice. However, we recommend to limit the number of steps to as few as possible (up to 5).

## Laboratory equipment

- heating block or water bath (range 37°C – 70°C)
- vortexer
- bench top centrifuge
- equipment for agarose gel electrophoresis
- gel extraction kit
- wet ice

## Cloning of modified blunt-ended DNA fragments

Genaxxon offers a special kit for blunt end cloning (M3457) which enables very fast and convenient ligation, cloning and transformation of your blunt-ended DNA.

If you don't want to use the blunt-end cloning kit, please follow the instructions below.

### Introduction

The cloning of blunt-ended DNA fragments into plasmid vectors is a common laboratory practice in molecular biology. These DNA fragments are typically generated by one of two methods: direct digestion with a restriction enzyme that produces a blunt end (e.g., *SmaI*, *StuI* or *PvuII*) or by using the controlled exonuclease activity of either Klenow or T4 DNA Polymerase.

In order to efficiently clone a blunt-ended fragment into a suitable vector, several requirements must be met. The vector should be dephosphorylated using Alkaline Phosphatase (M3033 or M3025), in order to minimize high backgrounds resulting from religated vector. The dephosphorylated vector must then be purified prior to ligation. Additionally, it may be necessary to modify the standard ligation buffer to include polyethylene glycol (which promotes macromolecular crowding) and low concentrations of ATP. Finally, high concentrations of T4 DNA Ligase and insert are recommended.

The description in this manual is a modification of a typical blunt-end cloning procedure to eliminate some of the requirements described above. The methods described here take advantage of the fact that *Taq* DNA Polymerase preferentially adds a single 3' A-deoxynucleotide to double-stranded DNA fragments by a non-template-dependent extension reaction. After a brief incubation of the blunt-ended DNA fragment with *Taq* DNA Polymerase and dATP, the resulting A-tailed fragment can be cloned using Genaxxon's pMBL-T/A Cloning Kit.

### DNA fragments generated by restriction digestion

The protocol below can be used for DNA fragments produced by restriction digestion with enzymes that generate blunt ends. The resulting 3'-tailed fragment can be ligated directly into the pMBL-T/A Vector without further purification using standard ligation conditions. For optimal transformation results, we recommend using no more than 1-2 $\mu$ l of the tailing reaction in the ligation mixture. The molar ratio of insert to vector should fall within an 8:1 to 1:8 range, however, a 1:1 molar ratio reflects optimal conditions with the inserts tested in these experiments. Using these conditions, 80-90% recombinants are typically obtained.

Addition of "A" to a blunt-end DNA fragment

- 1 – 2  $\mu$ L of DNA fragment (resuspended in water or TE buffer)
- 1  $\mu$ L of 10X *Taq* buffer
- 1  $\mu$ L of 25 mM MgCl<sub>2</sub>
- x  $\mu$ L of ATP solution (to a final conc. of 0.2 mM)
- 1  $\mu$ L of *Taq* Polymerase (5 Units / $\mu$ L)

add nuclease free water to a final volume of 10 $\mu$ L  
Incubate at 70°C for 30 minutes

### DNA fragments generated by thermostable polymerases

Blunt-ended DNA fragments can also be generated by some thermostable DNA polymerases (e.g., *Pfu* and *Tli* DNA polymerases) that have extensive 3'→5' exonuclease activity. A substantial proportion of the DNA fragments generated by these polymerases possesses blunt ends. The resulting fragments may be cloned by conventional methods or they may be modified by a second incubation with *Taq* DNA Polymerase in the presence of dATP (see protocol above) and then cloned into Genaxxon's pMBL-T/A vector. We recommend that the amplification reaction products be purified prior to modification to eliminate non specific reaction products (S5345: PCR Clean-up DNA Purification Kit). Following this procedure, 80-90% positive transformants can be typically obtained.

### References

1. Sambrook, J., Fritsch, E.F. and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.