Table of Contents

Description pT7Blue T-Vector 2

Kit Components 3

Insert Preparation 3
- Direct Cloning Without Purification 3
- Purification of Desired Product (Optional) 3

Ligation to pT7Blue T-Vector 4

Transformation 4

Screening 5
- Blue/White Phenotype 5
- Rapid Screening Method 6

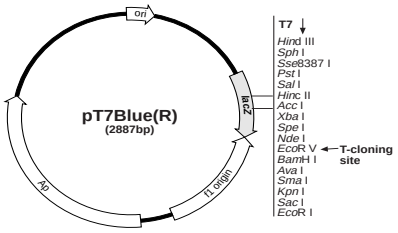
DNA Isolation and Sequencing 7

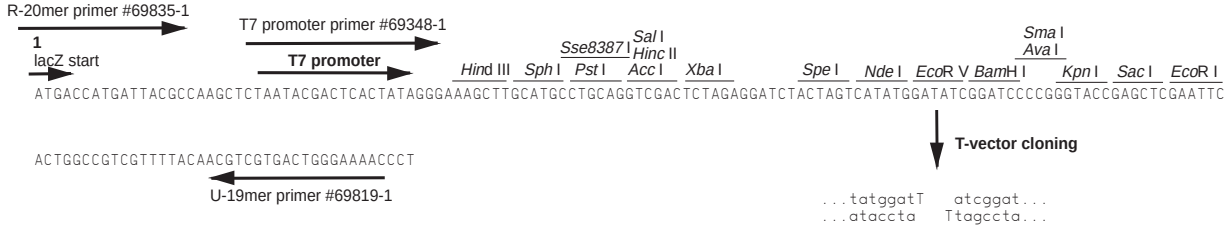
Description

pT7Blue T-Vector 69820-3

The pT7Blue T-Vector is designed for simpli•ed cloning of PCR[1] products. Many thermostable DNA polymerases, such as *Taq* DNA polymerase, leave single 3' A-nucleotide overhangs on their reaction products (Clark (1988) *Nucl. Acids Res.* **16**, 9677). These molecules can be ligated directly to a vector containing compatible single T-nucleotide overhangs (Marchuk et al. (1990) *Nucl. Acids Res.* **19**, 1154).

Novagen's pT7Blue vector has been speci•cally constructed for this application and has been prepared for T-cloning by digestion with EcoR V followed by the addition of single 3' dT residues at each end. The T-Vector is provided ready to ligate with DNA ampli•ed with any DNA polymerase that leaves single 3' dA overhangs. Target inserts having other types of ends, such as reaction products of *Pfu* or Vent (New England Biolabs) DNA polymerases or restriction fragments, can be easily prepared for T-cloning with Novagen's Single dA™ Tailing Kit (Cat. No. 69282-3).





[1] PCR is covered by U.S. Patent Numbers 4,683,195 and 4,683,202 owned by Hoffmann-La Roche.

Kit Components

- 2 µg pT7Blue T-Vector, 50 ng/µl
- 25 ng T-Vector Positive Control Insert, 2.5 ng/µl

Insert Preparation

Direct Cloning Without Purification

The need for PCR product puri•cation will be determined by the quality of the ampli•ed material (except in the case where PCR is being used to subclone an insert into the pT7Blue T-vector from ampicillin-resistant plasmids; see note below). If the PCR was very clean (i.e., the gel shows a clear, distinct band of the desired size with little extraneous material), a small sample (e.g., 1 µl) of the reaction can be added directly to the T-vector ligation. To avoid the possible generation of false positives by residual polymerase activity, the enzyme should be inactivated before removing a sample for ligation. This can be accomplished by extracting the reaction with 1 volume of chloroform:isoamyl alcohol (24:1). Add the CIAA, vortex for 1 minute, centrifuge at 12,000 g for 1 minute, and add up to 2 ml of the aqueous phase to the ligation. Precipitation is not necessary unless the PCR product is at a low concentration.

Purification of Desired Product (Optional)

(Other methods of partial puri•cation such as spin columns may be substituted.)

1. After the ampli•cation reaction add 100 µl of chloroform to remove the top oil phase, and then add 5 µl of 10X gel loading dye and load onto an agarose gel containing 0.5 µg/ml ethidium bromide. Run at 5–10 volts per cm. Load Novagen's PCR Markers (Cat. No. 69278-3) or other appropriate size markers in an adjacent lane.
2. Visualize the DNA band with a long wave UV light source and cut the band from the gel using a clean razor blade.
3. Recover the DNA from the gel slice using your favorite method. The Prep-A-Gene Kit (Bio Rad) has provided consistent recoveries for DNA larger than 200 bp. Resuspend the •nal product in a total volume of 10 µl (usually about 20 ng/µl).

Note: When using PCR to subclone into the pT7Blue T-Vector from ampicillin resistant plasmid templates, it is necessary to gel purify the fragment of interest to remove the original plasmid, which will transform very ef•ciently. As little as 10 pg of contaminating supercoiled plasmid can typically result in several hundred white colonies when using NovaBlue competent cells and the following protocols.

Ligation to pT7Blue T-Vector

1. For a standard reaction, 50 ng (0.03 pmol) of pT7Blue T-Vector is ligated with 0.2 pmol (50 ng of a 500 bp fragment) ampli•ed product in a volume of 10 µl. Assemble the following components in a 1.5-ml tube. The DNA Ligation Kit (69838-3) contains pretested components for this reaction.

1 µl	10X Ligase Buffer (200 mM Tris-HCl pH 7.6, 50 mM $MgCl_2$)
0.5 µl	100 mM DTT
0.5 µl	10 mM ATP
1 µl	50 ng/µl pT7Blue T-Vector
0.5 µl	T4 DNA Ligase (2–3 Weiss units)
X µl	Ampli•ed product (0.2 pmol)
Y µl	water
10 µl	

2. Add the ligase last and gently mix by stirring with a pipet tip. Incubate at 16 °C 2 hours to overnight.
3. To test the ef•ciency of ligation, use 2 µl (5 ng) of the T-Vector Positive Control Insert provided with the kit in place of the ampli•ed product in the above reaction. The molar ratio of insert to vector is about 5:1 under these conditions. The T-Vector Positive Control Insert is a 50 bp fragment having single 3' dA residues at each end.

Transformation

NovaBlue competent cells (69825-4) are provided in 0.2 ml aliquots. The standard transformation reaction calls for 20 µl cells, so each tube contains enough cells for 10 transformations. Cells can be refrozen at –70 °C and reused; however, transformation ef•ciencies will decline several-fold with each freeze-thaw cycle. For optimal performance, dispense the cells into desired aliquots after the initial thaw to avoid multiple cycles.

1. Thaw the required number of tubes of cells on ice and mix gently to assure that the cells are evenly suspended. Place the required number of 1.5 ml polypropylene microcentrifuge tubes on ice to pre-chill.
2. Pipet 20 µl aliquots of cells into the pre-chilled tubes.
3. (Optional) To determine transformation ef•ciency, add 1 µl (0.2 ng) Test Plasmid to one of the tubes containing cells. Gently flick the tube to mix. Plate only 5 µl of the •nal transformation mix (see below).
4. Add 1 µl of ligation reaction directly to the cells. Stir gently to mix.
5. Place the tubes on ice for 30 min.
6. Heat the tubes for exactly 40 seconds in a 42 °C water bath; do not shake.
7. Place on ice for 2 min.
8. Add 80 µl of room temperature SOC medium to each tube.

9. Shake at 200–250 rpm at 37 °C for 1 hour.

10. Spread 50 µl (see note) of each transformation on LB agar plates containing 50 µg/ml carbenicillin (or ampicillin) plus 15 µg/ml tetracycline. The tetracycline ensures that the selectable F' containing *lacZ* M15 is maintained and thus eliminates the background of non-recombinant white colonies that have lost the F'. For blue/white screening of recombinants, also include IPTG and X-gal in the LB agar. These can be pre-spread on the plates and allowed to soak in for about 30 min prior to plating. Use 35 µl of 50 mg/ml X-gal in dimethyl formamide and 20 µl 100 mM IPTG (in water) per 82 mm plate.

Note: The appropriate amount of transformation mixture to plate will vary with the ef• ciency of both the ligation and the competent cells. As little as 2 µl will yield several hundred transformants under highly ef• cient conditions (e.g., with cells giving >4 10^8 cfu/µg).

11. Let the plates sit on the bench for several minutes to allow excess liquid to be absorbed, and then invert and incubate overnight at 37 °C.

Screening

Blue/White Phenotype

The pT7Blue vector allows for blue-white screening of recombinants. The plasmid encodes a functional *lacZ* -peptide that complements the *lacZ* -fragment expressed by the host strain (NovaBlue). The resulting active -galactosidase can cleave the chromogenic substrate X-gal to yield a blue colony phenotype. Inserts are cloned into a modi•ed *Eco*R V site located within the -peptide coding region, which interferes with the function of the -peptide; therefore, recombinants appear white when plated on X-gal/IPTG indicator plates.

However, in addition to dark blue and white phenotypes, a light blue phenotype can be observed with relatively high frequency in vector + insert ligations, and to some extent in the absence of added insert. We have found that more than 90% of these light blue colonies generated from vector + insert ligations contain inserts of the expected size. Also note that the white colonies may develop a light blue center or "bullseye" appearance when grown to large sizes or during prolonged storage. Presumably a small amount of functional a-peptide is produced in these recombinants by means of ribosomal frameshifting, second site translational initiation, or as an a-peptide fusion protein. Light blue or white colonies that arise from self-ligated vector appear to be the result of an inefficient (but detectable) aberrant joining of single dT overhangs by T4 ligase by a mechanism that is unclear. These can be distinguished from authentic insert-containing white colonies by performing control ligations in the absence and presence of the T-Vector Positive Control Insert. The ligation with the insert should produce many more white colonies (~50–100X), although not necessarily a different proportion of white colonies, than the self-ligated vector. The following rapid PCR screening method also can be used to distinguish religated vector from

vector containing insert. Using the recommended primers, the religated vector should produce a 139 bp product, whereas recombinants containing the 50 bp T-Vector Positive Control insert should produce a 191 bp species.

Rapid Screening Method

Prior to growing colonies for plasmid isolation, the presence of the appropriate insert as well as its orientation can be determined using direct colony PCR. This additional step may be particularly helpful if a "dirty" (many extraneous bands), unpuri•ed PCR product was cloned. To determine insert orientation and size, 5 pmol (1 µl) of each vector-speci•c primer (T7 promoter primer, Cat. No. 69348-3, or R-20mer, Cat. No. 69835-3, are suitable 5' primers, and U-19mer primer, Cat. No. 69819-3, is a suitable 3' primer) is used with 5 pmol of one of the original PCR primers in two separate reactions. Alternatively, just the sequencing primers can be used in one reaction if insert orientation information is not desired.

By using the R-20mer primer and a 3' insert-speci•c primer, a fragment containing the T7 promoter and target insert in the correct orientation is ampli•ed. Target proteins can be expressed using in vitro transcription and translation (assuming that the target insert contains proper translation initiation and coding sequences; the upstream AUG and stop codon in the vector do not appear to interfere with initiation of translation with inserts tested thus far).

In addition, if one 5' phosphorylated primer was used for PCR, the products can be conveniently prepared for sequencing using Novagen's Strandase™ Kit (Cat. No. 69202-3) without the need for plasmid preparation. Novagen supplies highly purified, pretested phosphorylated primers for this application.

1. Pick a colony from an agar plate using a 200 µl pipet tip or sterile toothpick. Choose colonies that are at least 1mm in diameter and try to get as many cells as possible. If a "copy" of the colony is desired, touch the pipet tip to a plate before transferring the bulk of the colony to the tube in the next step.
2. Transfer the bacteria to a 1.5-ml tube containing 50 µl of sterile water. Vortex to disperse the pellet.
3. Place the tubes in boiling water or a heat block at 99 °C for 5 minutes to lyse the cells and denature DNases.
4. Centrifuge at 12,000 g for 1 min to remove cell debris.
5. Transfer 10 µl of the supernatant to a fresh 0.5-ml tube for PCR. Leave on ice until use.

6. Make a master reaction mix as follows:

   Per reaction:
   31.8 µl Nuclease-free water
   1 µl dNTP mix (10 mM each dATP, dCTP, dGTP, dTTP)
   1 µl 5' primer[2], 30 ng/µl, ~5 pmol/µl
   1 µl 3' primer, 30 ng/µl, ~5 pmol/µl
   5 µl 10X buffer (10X = 100 mM Tris-HCl pH 8.8 at 25 °C, 500 mM KCl, 15 mM $MgCl_2$, 1% Triton X-100)
   0.25 µl (1.25 U) AmpliTaq DNA polymerase (Perkin-Elmer)

Note: As an optional step, a "hot start" procedure can be used in which the cell lysate samples are prewarmed to 80 °C before the addition of the master mix.

Mix together the above components in a single tube using amounts corresponding to the number of reactions desired. (It is convenient to multiply the amounts by X.5, where X is the number of reactions, in order to account for pipetting losses.)

7. Add 40 µl of the master mix to each sample, mix gently, add 2 drops (~40 µl) of mineral oil, cap the tubes and put the samples in a thermocycler (Perkin-Elmer). Process for 35 cycles for 1 min at 94 °C, 1 min at 55 °C and 2 min at 72 °C, with a •nal extension at 72 °C for 5 min.

8. To analyze the reaction products, •rst remove the oil overlay by adding 100 µl of chloroform. Add 5 µl of 10X loading dye to the top aqueous phase and load 10-25 µl per lane on a 1% agarose gel containing 0.5 µg/ml ethidium bromide.

DNA Isolation and Sequencing

The pT7Blue vector can be used for double-stranded dideoxy sequencing of supercoiled plasmid DNA obtained using standard miniprep procedures, which is the simplest method and produces satisfactory results using T7 DNA polymerase, *Taq* DNA polymerase, Klenow fragment or reverse transcriptase as the enzyme. The plasmid has a very high copy number (pUC-based origin of replication) and suf•cient quality and quantities of DNA are produced from minipreps in the NovaBlue host. The following mini-prep protocol is a slight modi•cation of one presented in Sambrook et al. (1989) Molecular Cloning, A Laboratory Manual, Second Edition, Cold Spring Harbor Laboratory Press, and works very well for pT7Blue plasmids in NovaBlue.

1. Using a sterile loop, toothpick or pipet tip, transfer a well-isolated colony into 3 ml of LB broth supplemented with 50 µg/ml carbenicillin in a Falcon 2059 culture tube. Cap loosely and incubate with shaking at 37 °C 6 hours to overnight.

[2] Novagen's primers are designed and sold for use in the Polymerase Chain Reaction (PCR) process covered by patents owned by Hoffmann-La Roche. Use of the PCR process requires a license. A license for research may be obtained by purchase and use of authorized reagents and DNA thermocyclers from The Perkin-Elmer Corporation or by otherwise negotiating a license with Perkin-Elmer.

2. Transfer 1.5 ml of culture into a 1.5 ml microcentrifuge tube and centrifuge at 12,000 g for 1 min.

3. Remove the medium by aspiration, leaving the pellet as dry as possible.

4. Resuspend the cells in 100 µl of ice-cold 50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA. Pipet up and down to make sure that the pellet is completely suspended.

5. Add 200 µl of freshly prepared 0.2 N NaOH, 1% SDS. Mix by inversion and let sit on ice for 3 min.

6. Add 150 µl of ice-cold 3 M NaOAc, pH 5.2. Mix by inversion and leave on ice for 5 min.

7. Centrifuge at 12,000 g for 5 min. Transfer the clear supernatant to a fresh tube, avoiding the pellet, which tends to break up easily. Spin again if too much particulate matter remains in the supernatant.

8. Add 400 µl phenol:CIAA (1:1), vortex for 30 seconds, and centrifuge at 12,000 g for 1 min at room temperature.

9. Transfer the top aqueous phase to a fresh tube and add 800 µl ethanol. Vortex, leave at room temperature for 2 min, and centrifuge at 4 °C, 12,000 g for 5 min.

10. Decant the supernatant and add 400 µl ethanol to the pellet. Spin briefly, pour off the ethanol and allow the pellet to air dry in an inverted position for about 10 minutes.

11. Resuspend the pellet in 30 µl of TE buffer containing 20 µg/ml RNase and incubate at 37 °C for 15 min. Expect a yield of about 5–8 µg plasmid DNA.

12. At this point the DNA can be analyzed by restriction digestion, etc., but it should be further processed to remove RNA breakdown products before sequencing the double-stranded plasmid. This can be simply accomplished by precipitation with polyethylene glycol. Add 10 µl of 30% PEG-8000, 1.5 M NaCl (prepare this solution from autoclaved stocks of PEG and NaCl to avoid possible DNase contamination, e.g., 30 ml 50% PEG + 15 ml 5 M NaCl + 5 ml sterile water for 50 ml), vortex thoroughly, and incubate on ice for 20–60 min.

13. Centrifuge at 12,000 g at 4 °C for 10 min. Carefully remove the supernatant, leaving the small transparent DNA pellet behind. Rinse the pellet successively with 70% ethanol and then 100% ethanol as above, and let air dry.

14. Resuspend the DNA in 20 µl TE. The plasmid is now suitable for alkali denaturation and double-stranded sequencing.

Note: At this point the plasmid DNA is not suitable for in vitro transcription or translation because of residual RNase activity. The RNase is removed by extracting twice with 1 vol TE-buffered phenol:CIAA (1:1) and once with 1 vol CIAA, followed by precipitation with ethanol after addition of 0.1 vol 3 M NaOAc. Rinse the pellet as in Step 13 and resuspend in RNase-free TE buffer.

As an alternative, it is possible to prepare single-stranded DNA template from pT7Blue recombinants because the plasmid carries the phage f1 origin of replication. The f1 origin in pT7Blue(R) is oriented such that the single-stranded DNA produced will anneal with the T7 promoter primer. The required helper phage (strain R408 or M13KO7) and protocols for infection and DNA isolation are available from a number of commercial suppliers. (The NovaBlue host strain carries an F' and is therefore suitable for helper phage infection.) Approximately 5 pmol of each primer is recommended for each set of sequencing reactions, whether double- or single-stranded templates are used. Detailed protocols for sequencing with double-stranded and single-stranded templates are available from many manufacturers of sequencing kits. The following references are also useful.

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Hattori, M. and Sakaki, Y. (1986) *Anal. Biochem.* **152**, 232.

Mierendorf, R. and Pfeffer, D. (1987) *Meth. Enzymol.* **152**, 556–562.

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