PCR Product Cloning Using T Vectors

Usage

Ligate PCR products generated with proofreading or nonproofreading DNA polymerases into a T vector, followed by bacterial transformation.

Abstract

T vectors are linear vectors with a 3´ terminal thymidine at each end. These single 3´-T overhangs allow efficient ligation of a PCR product into the plasmids by preventing recircularization of the vector and providing a compatible overhang for PCR products with a single deoxyadenosine at the 3´-ends. With an additional A tailing step, a PCR product generated by a proofreading DNA polymerase can also be cloned into a T vector.

Reagents

- GoTaq® Flexi DNA Polymerase
- 25mM MgCl₂, supplied with GoTaq® DNA Polymerase
- Purified DNA fragment, 1-4.4 µl
- 5X GoTaq® Reaction Buffer (Colorless or Green), 2 µl
- 1mM dATP (0.2mM final concentration), 2 µl
- GoTaq® Flexi DNA Polymerase (5 u/µl), 1 µl
- 25mM MgCl₂ (1.5mM final concentration), 0.6 µl
- Nuclease-free water

Instructions

A-Tailing Reaction for Blunt-Ended Products (Optional)

1. If a proofreading DNA polymerase was used for amplification and you want to clone into a T vector, an adenosine residue must be added onto the PCR product. This can be accomplished by incubating the DNA fragment with dATP and a nonproofreading DNA polymerase, which will add a single 3´ A residue. Blunt DNA fragments resulting from restriction enzyme digestion can also be cloned into T vector after adding an adenosine residue.

Set up the following reaction in a thin-walled PCR tube:

1. Purified DNA fragment, 1-4.4 µl
2. 5X GoTaq® Reaction Buffer (Colorless or Green), 2 µl
3. 1mM dATP (0.2mM final concentration), 2 µl
4. GoTaq® Flexi DNA Polymerase (5 u/µl), 1 µl
5. 25mM MgCl₂ (1.5mM final concentration), 0.6 µl
6. Nuclease-free water to, 10 µl
7. Incubate at 70°C for 15-30 minutes in a water bath or thermal cycler.
8. After the tailing reaction is finished, 1-2 µl can be used without further cleanup for ligation with pGEM®-T or pGEM®-T Easy Vector Systems.

Vector:Insert Ratio

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1. After the insert DNA has been prepared for ligation, estimate the concentration by comparing the staining intensity with that of DNA molecular weight standards of known concentrations on an ethidium bromide-stained agarose gel. If the vector DNA concentration is unknown, estimate the vector concentration by the same method. Test various vector:insert DNA ratios to determine the optimal ratio for a particular vector and insert. In most cases, either a 1:1 or a 1:3 molar ratio of vector:insert works well. The following example illustrates the calculation of the amount of insert required at a specific molar ratio of vector.

\[
\text{ng of insert} = \frac{\text{[ng of vector } \times \text{ size of insert (in kb)]}}{\text{size of vector (in kb) } \times \text{ molar amount of (insert } + \text{ vector)}}
\]

Ligation

1. Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect contents at the bottom of the tube.
2. Set up ligation reactions as described below.
3. Vortex the 2X Rapid Ligation Buffer vigorously before each use.
4. Use 0.5ml tubes known to have low DNA-binding capacity.

Standard reaction

1. 2X Rapid Ligation Buffer 5 µl
2. pGEM®-T or pGEM®-T Easy Vector (50 ng) 1 µl
3. PCR product X µl
4. T4 DNA Ligase (3 Weiss units/µl) 1 µl
5. Nuclease-Free Water to a final volume of 10 µl

Positive Control

1. 2X Rapid Ligation Buffer 5 µl
2. pGEM®-T or pGEM®-T Easy Vector (50 ng) 1 µl
3. Control Insert DNA 2 µl
4. T4 DNA Ligase (3 Weiss units/µl) 1 µl
5. Nuclease-Free Water to a final volume of 10 µl

Background Control

1. 2X Rapid Ligation Buffer 5 µl
2. pGEM®-T or pGEM®-T Easy Vector (50 ng) 1 µl
3. PCR product
4. T4 DNA Ligase (3 Weiss units/µl) 1 µl
5. Nuclease-Free Water to a final volume of 10 µl
6. Mix the reactions by pipetting.
7. Incubate the reactions for 1 hour at room temperature.
8. Alternatively, incubate the reactions overnight at 4°C for the maximum number of transformants.

Transformation

1. LB plates with ampicillin.
2. 10 g/L Bacto®-tryptone
3. 5 g/L Bacto®-yeast extract
4. 5 g/L NaCl
5. Adjust the pH to 7.5 with NaOH.
6. Add 15g of agar per liter.
7. Autoclave to sterilize.
8. Allow the autoclaved medium to cool to 55°C.
9. Add ampicillin to a final concentration of 50-125 µg/ml.
10. Add IPTG to a final concentration of 0.5 mM and X-gal to a final concentration of 80 µg/ml.
11. Alternatively, spread 100 µl of 100 mM IPTG and 20 µl of 50 mg/ml X-Gal over the surface of an LB-ampicillin plate.

12. Allow to absorb for 30 minutes at 37°C prior to use.

**Preparation of SOC medium (100ml)**

1. 2.0 g Bacto®-tryptone
2. 0.5 g Bacto®-yeast extract
3. 1 ml 1M NaCl
4. 0.25 ml 1M KCl
5. 1 ml 2M Mg2+ stock, filter-sterilized
6. 1 ml 2M glucose, filter-sterilized
7. Add Bacto®-tryptone, Bacto®-yeast extract, NaCl and KCl to 97ml of distilled water.
8. Stir to dissolve.
9. Autoclave and cool to room temperature.
10. Add 2 M Mg2+ stock and 2 M glucose, each to a final concentration of 20 mM.
11. Bring the volume to 100 ml with sterile, distilled water. The final pH should be 7.0.
12. Centrifuge the ligation reactions briefly.
13. Add 2 µl of each ligation reaction to a sterile 1.5 ml microcentrifuge tube on ice.
14. Prepare a transformation control tube with 0.1 ng of an uncut plasmid. pGEM®-T Vectors are not suitable for the transformation control as they are linear, not circular.

Note: In our experience, the use of larger (17 × 100mm) polypropylene tubes (e.g., BD Falcon Cat.# 352059) has been observed to increase transformation efficiency. Tubes from some manufacturers bind DNA, thereby decreasing the colony number, and should be avoided.

16. Place the high-efficiency JM109 Competent Cells in an ice bath until just thawed (5 minutes).
17. Mix cells by gently flicking the tube.
18. Carefully transfer 50 µl of cells to the ligation reaction tubes prepared in Step 2. Use 100 µl of cells for the transformation control tube.
19. Gently flick the tubes, and incubate on ice for 20 minutes.
20. Heat-shock the cells for 45-50 seconds in a water bath at exactly 42°C. Do not shake. Immediately return the tubes to ice for 2 minutes.
21. Add 950 µl of room temperature SOC medium to the ligation reaction transformations and 900 µl to the tranformation control tube.
22. Incubate for 1.5 hours at 37°C with shaking (~150 rpm).
23. Plate 100 µl of each transformation culture onto duplicate LB/ampicillin/IPTG/X-Gal plates. For the transformation control, a 1:10 dilution with SOC is recommended prior to plating.
24. Incubate plates overnight at 37°C. Select white colonies.

**Calculation of Transformational Efficiency**

1. For every transformation with competent cells, we recommend performing a transformation control experiment using a known quantity of a purified, supercoiled plasmid DNA. Calculate the transformation efficiency as described below.
2. Transformation efficiency (cfu/µg) = (cfu on control plate ÷ ng of supercoiled vector plated) × (10³ ng/µg) × final dilution factor
3. cfu = colony forming units

Citation:

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