05_04_pGEM-T-Easy-Vector-System

DONNERSTAG, 5.8.2021

Goal-Setting

- Cloning of an insert and transformation into competent cells according to

pGEM T and pGEM T Easy Vector Systems TM042 (1).pdf

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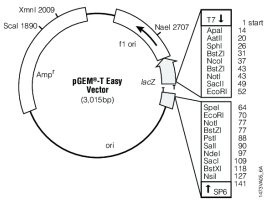
Promega

5.D. pGEM®-T Easy Vector Map and Sequence Reference Points



pGEM®-T Easy Vector sequence reference points:

T7 RNA polymerase transcription initiation site	1
multiple cloning region	10–128
SP6 RNA polymerase promoter (–17 to +3)	139–158
SP6 RNA polymerase transcription initiation site	141
pUC/M13 Reverse Sequencing Primer binding site	176–197
lacZ start codon	180
lac operator	200–216
β-lactamase coding region	1337–2197
phage f1 region	2380–2835
lac operon sequences	2836–2996, 166–395
pUC/M13 Forward Sequencing Primer binding site	2949–2972
T7 RNA polymerase promoter (–17 to +3)	2999–3

Note: Inserts can be sequenced using the pUC/M13 Forward Primer (Cat.# Q5601) or pUC/M13 Reverse Primer (Cat.# Q5421).

Note: A single digest with BstZI, EcoRI (Cat.# R6011) or NotI (Cat.# R6431) will release inserts cloned into the pGEM®-T Easy Vector. Double digests can also be used to release inserts. Isoschizomers of BstZI include EagI and Eco52I.

vector sequence: https://www.promega.de/products/pcr/pcr-cloning/pgem-t-easy-vector-systems/~/media/D30BDACB5C214995AAD6D78C779CE2B1.ashx
3015 bp

pGEM T easy vector.dna

Terms / abbreviations

- cPCR = Colony polymerase chain reaction
- RT = Room temperature

Risk areas

- None

Required materials and / or information

- Chemicals:
  - Chemical competent cells (E. coli C3040I)
  - S.O.C. Medium, Thermo Fisher Scientific or self-made
- Materials:
  - 0.5 mL microcentrifuge tubes, VWR
  - 1.5 mL tubes, Eppendorf
  - Agar plates (Ampicillin 100 µg/mL)
  - Ice
  - pGEM®-T Easy Vector System I, Promega , containing:
    - 1.2 µg pGEM®-T Easy Vector (50 ng/µL)
    - 12 µL Control Insert DNA (4 ng/µL)
    - 100 U T4 DNA Ligase
    - 200 µL 2x Rapid Ligation Buffer, T4 DNA Ligase
  - Pipettes, Eppendorf
  - Polypropylene tube or 1.5 mL microcentrifuge tube
  - Timer
  - Wizard® SV Gel and PCR Clean-Up System, Promega

Templates, devices, software

- ThermoMixer C, Eppendorf

Preliminary work

- 03_01_Thermofisher-protocol-for-TdT-tailing-reaction
- 03_02_Heat-inactivation-of-TdT-reaction
- 03_04_PCR-for-ssDNA-samples
- 05_01_Preparation-of-S.O.C.-medium

Operation

- Briefly centrifuge the pGEM®-T or pGEM®-T Easy Vector and Control Insert DNA tubes to collect the contents at the bottom of the tubes
- Set up ligation reactions as described below
  - Note: Use 0.5 mL tubes known to have low DNA-binding capacity
- Vortex the 2x Rapid Ligation Buffer vigorously before each use
- Mix the reactions by pipetting. Incubate the reactions for 1 h at RT. Alternatively, if the maximum number of transformants is required, incubate the reactions overnight at 4 °C.

Ligation Reaction

	A	B	C	D
1	Component	Standard Reaction [µL]	Positive Control [µL]	Background Control [µL]
2	2x Rapid Ligation Buffer, T4 DNA Ligase	5	5	5
3	pGEM®-T Easy Vector (50 ng)	1	1	1
4	PCR Product	x	-	-
5	Control Insert DNA	-	2	-
6	T4 DNA Ligase (3 Weiss units/µL)	1	1	1
7	nuclease free water to final v. of	10	10	10

*Molar ratio of PCR product: vector may require optimization

Notes:

- Use only the T4 DNA Ligase supplied with this system to perform pGEM®-T and pGEM®-T Easy Vector ligations. Other commercial preparations of T4 DNA ligase may contain exonuclease activities that may remove the terminal deoxythymidines from the vector.
- 2x Rapid Ligation Buffer contains ATP, which degrades during temperature fluctuations. Avoid multiple freeze-thaw cycles and exposure to frequent temperature changes by making single-use aliquots of the buffer.
- Longer incubation times will increase the number of transformants. Generally, incubation overnight at 4 °C will produce the maximum number of transformants.
- An aliquot of the PCR reaction should be analyzed on an agarose gel before use in the ligation reaction to verify that the reaction produced the desired product. The PCR product to be ligated can be gel-purified or purified directly from the PCR amplification using the Wizard® SV Gel and PCR Clean-Up System. Clean-up of reactions prior to ligation is recommended to remove primer-dimers or other undesired reaction products and to improve ligation efficiency. Exposure of PCR products to shortwave ultraviolet light should be minimized in order to avoid the formation of pyrimidine dimers.

Optimizing insert: Vector molar ratios

- The pGEM®-T and pGEM®-T Easy Vector Systems have been optimized using a 1:1 molar ratio of the Control Insert DNA to the vectors. However, ratios of 8:1 to 1:8 have been used successfully. If initial experiments with the PCR product are suboptimal, ratio optimization may be necessary. Ratios from 3:1 to 1:3 provide good initial parameters. The concentration of PCR product should be estimated by comparison to DNA mass standards on a gel or by using a fluorescent assay. The pGEM®-T and pGEM®-T Easy Vectors are approximately 3 kb and are supplied at 50 ng/µL. To calculate the appropriate amount of PCR product (insert) to include in the ligation reaction, use the following equation:
  [(ng of vector × kb size of insert)/ kb size of vector]× insert: vector molar ratio = ng of insert

Transformation

1. Agar Plates should be stored at RT.
2. Centrifuge the tubes containing the ligation reactions to collect the contents at the bottom. Add 2 µL of each ligation reaction to a sterile (17 × 100 mm) polypropylene tube or a 1.5 mL microcentrifuge tube on ice. Set up another tube on ice with 0.1 ng uncut plasmid for determination of the transformation efficiency of the competent cells.
3. Remove tube(s) of frozen E. coli C3040I chemically competent cells from storage and place them in an ice bath until just thawed (about 5 min). Mix the cells by gently flicking the tube. Avoid excessive pipetting, as the competent cells are extremely fragile.
4. Carefully transfer 50 µL of cells into each tube prepared with ligation reaction (use 100 µL of cells for determination of transformation efficiency).
5. Gently flick the tubes and place them on ice for 20 min.
6. Heat-shock the cells for 45–50 s in a water bath at exactly 42 °C (do not shake).

7. Immediately return the tubes to ice for 2 min.
8. Add 950 µL room-temperature S.O.C. medium to the tubes containing cells transformed with ligation reactions and 900 µL to the tube containing cells transformed with an uncut plasmid (LB broth may be substituted, but colony number may be lower).
9. Incubate for 1.5 h at 37 °C with shaking (~150 rpm).
10. Plate 100 µL of each transformation culture onto LB Ampicillin plates. For the transformation control, a 1:10 dilution with S.O.C. medium is recommended for plating. If a higher number of colonies is desired, the cells may be pelleted by centrifugation at 1000 g for 10 min, resuspended in 200 µL of S.O.C. medium, and 100 µL plated on each of two plates.
11. Incubate the plates overnight (16–24 h) at 37 °C. If 100 µL is plated, approximately 100 colonies per plate are routinely seen using competent cells that are 1 × 108 cfu/µg DNA

Example of transformation efficiency calculation

- After 100 µL of competent cells are transformed with 0.1 ng of uncut plasmid DNA, the transformation reaction is added to 900 µL of S.O.C. medium (0.1 ng DNA/mL). From that volume, a 1:10 dilution with S.O.C. medium (0.01 ng DNA/mL) is made, and 100 µL are plated on two plates (0.001 ng DNA/100 µL). If 200 colonies are obtained (average of two plates), what is the transformation efficiency?
- 200 cfu/0.001 ng = 2 × 105 cfu/ng = 2 × 108 cfu/µg DNA

Troubleshooting

- Expected heights of the bands after cPCR:
    - Uncut vector: 263 bp (as it is circular, it will run farther than linear DNA, so band will be lower)
    - Re-ligated vector: 264 bp
    - Vector with control insert: 805 bp
    - Vector with AT-rich insert: 263 + ~insert length bp

Follow-up work

- None