pGM-T Ligation Kit

For direct cloning of PCR products generated by *Taq* DNA polymerases

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pGM-T Ligation Kit

Cat. no. SC10

Kit Contents

Contents	SC1001 (20 reac)
pGM-T Vector (50ng/μl)	20 μl
T4DNA Ligase (3U/μl)	20 μl
10× T4 DNA Ligation Buffer	30 μl
2×T4 Rapid Ligation Buffer	100 μl
Control Fragment (700 bp, 50ng/μl)	10 μl
ddH2O	1 ml
Handbook	1

Storage

The reagents (containing vector and ligation reagents) are stored at -20°C. Subdivision could be taken to avoid repeated freezing and thawing.

Introduction

The pGM-T Vectors are linearized vectors with a single 3´-terminal thymidine at both ends. The T-overhangs at the insertion site greatly improve the efficiency of ligation of PCR products by preventing recircularization of the vector and providing a compatible overhang for PCR products generated by certain thermostable polymerases.

The pGM-T Vectors are high-copy-number vectors containing T7

and SP6 RNA polymerase promoters flanking a multiple cloning region within the α-peptide coding region of the enzyme β-galactosidase. Insertional inactivation of the α-peptide allows identification of recombinants by blue/white screening on indicator plates.

Important Notes

1. It's necessary to use the control fragment during the transformation process, to confirm the reasons of the problems coming out in the experiment.
2. It's suggested to leave part of ligation product. If the last steps appeared problem, you could remedy quickly and couldn't repeat the ligation step.
3. The bacterium volume adding on containing antibiotic SOB or LB solid culture agarose could be adjusted according to the experiment. If the quantity of transformation DNA was large, the bacterium volume could be less than 100ul; contrarily, the bacterium volume could be 200-300 μl. If it's estimated that the clone was less, it could centrifuge 4,000rpm for 2 min firstly, and then take out part of culture medium. Mix the remaining medium and bacterium, then extract suitable bacterium to add on containing antibiotic SOB or LB solid culture agarose. Other remaining bacterium could store at 4℃. If the transformation bacterium colonies was less in the next day, it could extract stored bacterium to add on new containing antibiotic SOB or LB solid culture agarose.

Protocol

1. If the 3'-terminal including the 3'-A overhangs, start from step 3 directly.

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 PCR cleanup & gel extraction system. Clean-up of reactions prior to ligation is recommended to remove primer dimers or other undesired reaction products, and to improve ligation efficiency.

2. Prepare an A-addition reaction according to the following table.

Component	Volumen/reaction
PCR product (blunt)	15 µl
A-addition buffer	4 µl
Taq DNA polymerase	1 µl

Reaction condition: at 72°C for 20-30 min. The product after A-addition procedure can be used in ligation reaction.

3. The vector must be melted on the ice. Don't freeze and thaw repeatedly. Centrifugate the tube with vector shortly tube to remove drops from the inside of the lid.

4. Add all kinds of component in the asepsis tube as following: **The mol ratio of vector and insert fragment must be controlled in 1:3 ~ 1:8. More fragments will disturb ligation reaction.**

	Using 10 x Ligation Buffer		Using 2x Ligation Rapid Buffer	
Contents in Ligation system	Reaction system	Control system	Reaction system	Control system
PCR fragment (added A)	Xμl	--	X μl	--
Control fragment (700 bp, 50 ng/μl)	--	1 μl	--	1 μl
pGM-T Vector (about 50 ng/μl)	1 μl	1 μl	1 μl	1 μl
10×T4 DNA Ligation Buffer	1 μl	1 μl	--	--
2× T4 DNA Rapid Ligation Buffer	--	--	5 μl	5 μl
T4 DNA Ligase (3 U/μl)	1 μl	1 μl	1 μl	1 μl
ddH2O	Up to 10 μl	Up to 10 μl	Up to 10 μl	Up to 10 μl

Note: There are two kinds of buffer in the kit, 10× T4 DNA Ligation Buffer and 2× T4 DNA Rapid Ligation. Don't add two buffers in one tube.

5. Mix the solution in the tube gently, then centrifuges shortly. Incubate the tube with mixed solution at 22-26°C for 1-2 hours using 10×T4 DNA Ligation Buffer or incubate at 16°C overnight. If use2×T4 DNA Rapid Ligation Buffer, incubatethe tube with mixed solution at 22-26°C for 5-10 min. If it lasts for more than 15 min, the ligation efficiency would reduce. After reaction, place the tube on the ice.

Note: It's suggested that incubate the tube with mixed solution at 16°C overnight using 10× T4 DNA Ligation Buffer.

6. Transformation

a. Prepare agar plates for transformation.

Add 16 µl IPTG (50 mg/ml) and 40 µl X-gal (20 mg/ml) on agar plate surfacewhich contain corresponding antibiotic. Smear completely using a sterile bent glass rod or a specialized spreader. Then put that plate at 37°C for 1-3 hours with no light.

b. Transform according to the protocol of electrical or chemical transformation

7. Detection

a. General detection: pick the white colony, inocluate 1-5 ml liquid LB culture medium containing 50-100 µg/ml ampicillin, and culture at 37°C overnight with shaking. Save bacterium strain and extract plasmid. To detect whether the fragment has inserted rightly using PCR or enzyme cutting.

b. Quick detection: to detect whether the fragment has inserted rightly using bacterium PCR directly (colony PCR).

c. Sequencing: sequence the fragment after general or quick detection.

1. Add the following components to a nuclease-free micro-centrifuge tube.
   - 2 µl oligo $(dT)_{12-18}$ (10 µM), or 2 µl random primers (10 µM), or 2 pmol gene-specific primers;
   - 1-5 µg total RNA, or 50-500 ng mRNA;
   - 2 µl dNTP mixture (10 mM total, with neutral PH value);
   - Add RNase-free ddH_2O up to 15 µl.
2. Heat at 70 °C for 5 min, and place the tube immediately on ice for 2 min. Centrifuge briefly and then add 4 µl 5× First-Strand Buffer (with DTT).

   Optional Step: If the amount of starting template is less than 50 ng, 0.5-1 µl RNasin (40 U/µl) should be added.
3. Add 1 µl M-MLV and mix gently by pipetting; when using random primers, incubate the tube at 25°C for 10 min.
4. Incubate at 42 °C for 50 min.
5. Heat the sample to 95°C for 5 min to inactivate enzyme. Cool the sample on ice for downstream experiments or store at -20°C immediately.

If the RNase H is needed, perform the step 6. Or, proceed to step 7 directly.

6. Add 1 µl RNase H (2 U), incubate at 37°C for 20 min to degrade RNA. Then heat the sample to 95°C for 5 min to inactivate RNase H.
7. Dilute the reaction system to 50 µl in RNase-free ddH_2O. Take 2-5 µl for PCR amplification.

PCR Amplification

Take 10% of the first-strand cDNA synthesis reaction mixture (2 µl) for PCR; increasing amount of cDNA synthesis products not lead to highly efficient DNA amplification and inhibitors presenting in the reverse-transcription products may inhibit the PCR.

1. Prepare reaction mixture by adding the following components to a microcentrifuge tube.

Reagents	Volume
10x PCR Buffer (200 mM Tris-HCL (PH 8.4), 500 mM KCl)	5 µl
50 mM $MgCl_2$	1.5 µl
dNTP (2.5 mM each)	1 µl
Primer 1 (10 µM)	1 µl
Primer 2 (10 µM)	1 µl
Taq DNA Polymerase (5 U/µl)	0.4 µl
cDNA (synthesis reaction mixture)	2 µl
ddH_2O	Up to 50 µl

Note: To obtain the optimal result, the concentration of $MgCl_2$ should be optimized for individual template-primer combination.

2. Mix gently and overlay the reaction with one or two drops (~50 µl) of nuclease-free mineral oil to prevent evaporation and condensation. (Mineral oil is not necessary if the thermo cycler has been equipped with hot lid.)
3. Denature at 94°C for 2 min.
4. Set 15-40 PCR cycles. The conditions of annealing and denaturation should be optimized for individual primer and template.