QuickClone™ 2.1 TA Cloning Kit

Cat. No: PGK202-A

CONTENTS

COMPONENTS OF THE KIT

Component	PGK202-A 20 Rxns
pCR2.1, linearized 25 ng/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8	5 × 10 µL
T4 DNA Ligase 5.0 Weiss units/µL	25µl
5X T4 DNA Ligase Buffer 5X T4 DNA Ligase Buffer (50 mM Tris-HCl, pH 7.6, 50 mM $MgCl_2$, 5 mM ATP, 5 mM dithiothreitol, 25 % (w/v) polyethylene glycol-8000)	200µl
10X PCR Buffer 100 mM Tris-HCl, pH 8.3 (at 42°C) 500 mM KCl 25 mM $MgCl_2$ 0.01% gelatin	100µl
50 mM dNTPs 12.5 mM dATP 12.5 mM dCTP 12.5 mM dGTP 12.5 mM dTTP (adjusted to pH 8.0)	10µl
Control DNA Template 0.1 µg/µL in 10 mM Tris-HCl, 1 mM EDTA, pH 8	10µl
Sterile Water Deionized, autoclaved water	1 mL
Control PCR Primers 0.1 µg/µL each in 10 mM Tris-HCl, 1 mM EDTA, pH 8	10 µL

STORAGE

All components of the kit should be stored at -20 °C.

DESCRIPTION

The Purgene's QuickClone™ 2.1 TA Cloning Kit is a TA system for direct one-step cloning of PCR products with 3'-dA overhangs. The high-quality TA cloning vector pCR2.1 is ready to use for efficient ligation with PCR products providing high cloning yields and low background

CLONING PRINCIPLE

The QuickClone™ 2.1 TA Cloning Kit takes advantage of the terminal transferase activity of *Taq* DNA polymerase and other non-proofreading thermostable DNA polymerases. Such enzymes add a single 3'-A overhang to both ends of the PCR product. The structure of these PCR products favors direct cloning into a linearized cloning vector with single 3'-ddT overhangs. Such overhangs at the vector cloning site not only facilitate cloning, but also prevent the recircularization of the vector. As a result, more than 90% of recombinant clones contain the vector with an insert. Recombinant clones are selected based on blue/white screening.

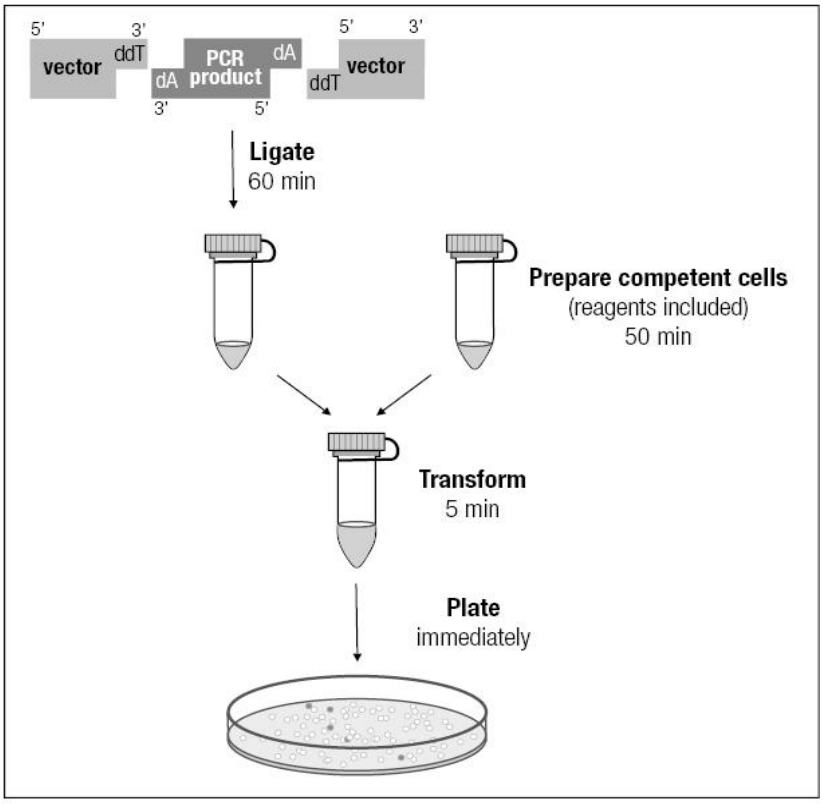


Fig. 1. PCR product cloning with QuickClone™ 2.1 TA Cloning Kit.

IMPORTANT NOTES

- Include final extension step in the PCR cycling protocol to ensure efficient 3'-dA tailing of the PCR product. The final extension step prolonged to 20-30 minutes generally yields 3-4 fold higher numbers of recombinant clones.
- Thoroughly mix every vial before use.
- The QuickClone™ 2.1 TA Cloning Kit is compatible with all PCR buffers.
- Gel-analyze the PCR product for specificity and yield before cloning.
- Specific PCR products of <1 kb appearing as one discrete band on the gel can be used for ligation directly from PCR reaction mixture without any purification.
- Do not use more than 4 µL of unpurified PCR product in the ligation reaction. Excess salts from the PCR reaction mixture may reduce the efficiency of the cloning procedure.
- Gel purification of the PCR product is recommended to increase the number of recombinants containing full-length inserts in following cases:
  – PCR product is longer than 1 kb;
  – PCR product is contaminated with non-specific PCR products;
  – PCR product is contaminated with primer-dimers;
  – PCR template contains β-lactamase (ampicillin resistance) gene, which may result in background colonies on LB-ampicillin agar plates.
- For efficient cloning of gel-purified DNA fragments, it is important to avoid DNA damage by ethidium bromide and UV light. Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When using a short-wavelength (254-312 nm) light-box, limit DNA exposure to UV to a few seconds. Keep the gel on a glass plate or on a plastic plate during UV illumination. Alternatively, use dyes, like crystal violet, to visualize DNA in ambient light.
- Use the formula below to estimate the amount of PCR product needed to ligate with 50 ng (20 fmoles) of pCR2.1 vector:

$$x \text{ ng PCR product} = \frac{(y \text{ bp PCR product})(50 \text{ ng pCR2.1 vector})}{(\text{size in bp of the pCR2.1 vector: } \sim 3900)}$$

  where x ng is the amount of PCR product of y base pairs to be ligated for a 1:1 (vector:insert) molar ratio.
- In general, 0.5–1.0 µL of a typical PCR sample with an average insert length (400–700 bp) will give the proper ratio of 1:1 (vector:insert), with efficient ligation. If you are concerned about the accuracy of your DNA concentrations and want to increase efficiency, then do a second ligation reaction at a ration 1:3.
- Do not use more than 2–3 µL of the PCR sample in the ligation reaction because salts in the PCR sample may inhibit T4 DNA Ligase.

CLONING PROTOCOL

For optimal ligation efficiencies, we recommend using fresh (less than 1 day old) PCR products. The single 3' A-overhangs on the PCR products will be degraded over time, reducing ligation efficiency.
Take care when handling the pCR2.1 vector as loss of the 3' T-overhangs will cause a blunt-end self-ligation of the vector and subsequent decrease in ligation efficiency.

Ligation

1. Set up the ligation reaction:

Component	Volume
pCR2.1 Vector, (25 ng/µL)	2 µL
5X Ligation Buffer	2 µL
PCR product	variable*
Nuclease-free Water	to 9 µL
T4 DNA Ligase	1 µL
Total volume	**10 µL**

* Do not use more than 4 µL of unpurified PCR product in the ligation reaction. Excess salts from the PCR reaction mixture may reduce the efficiency of the cloning procedure.

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the ligation mixture at room temperature (22°C) for 15 minutes. If maximal number of transformants is required, incubate overnight at 4 °C.

Note. During the ligation prepare competent *E. coli* cells using the provided set of solutions for preparation of competent cells –Bacterial Transformation Kit. To enable blue/white screening, choose only strains having lacZ∆M15 mutation, for example, XL1-Blue, ER1727, JM109 or other. Refer to transformation protocols on next page.

3. Use 1 µL of the ligation mixture directly for bacterial transformation.

Note. Keep the ligation mixture at -20 °C if transformation is postponed. Thaw on ice and mix carefully before transformation.

Cloning efficiency can be optimized by changing incubation time and altering the vector to insert ratio. In the tables below, the ligation reactions were performed using a 1:1 vector to insert ratio (Table 1) or using a 1:3 vector to insert ratio, which was achieved by reducing the pCR 2.1 vector concentration to 25ng (Table 2).

Table 1 - Vector to insert Ratio 1:1			Table 2 - Vector to insert Ratio 1:3		
Time	**Total Colonies**	**% White**	**Time**	**Total Colonies**	**% White**
15 min	312 ± 137	75± 6	15 min	141 ± 43	75± 16
30 min	315 ± 23	70± 4	30 min	160 ± 81	74± 8
60 min	312 ±141	75± 9	60 min	176 ±36	82± 3

Transformation

All common *E. coli* laboratory strains can be used for transformation. To enable blue/white screening, choose only strains having lacZΔM15 mutation, for example, XL1-Blue, ER1727, JM109 or other. Typical transformation efficiencies are more than 10^7 transformants per µg of supercoiled plasmid DNA.

CONTROL EXPERIMENT

We recommend performing the control reactions the first time you use the kit to help you evaluate results. Performing the control reactions involve producing a control 2.1the reagents included in the kit and using this product in a ligation reaction.

1. Use Taq Polymerase and the protocol below to amplify the control PCR product.

Component	Volume
Control DNA Template (100 ng)	1 µL
10X PCR Buffer	5 µL
50 mM dNTPs	0.5 µL
Control PCR Primers	1 µL
Water	41.5 µL
Taq Polymerase (1 unit/µL)	1 µL
Total volume	**50 µL**

2. Amplify using the cycling parameters below:

Step	Time	Temperature	Cycle
Denaturation	1 minute	94°C	25
Annealing	1 minute	55°C	
Extension	1 minute	72°C	
Final Extension	7 minute	72°C	1

3. Remove 10 µL from the reaction and analyze by agarose gel electrophoresis. A discrete 700 bp band should be visible. Proceed to the Control Ligation Reaction.

Control Ligation Reaction

1. Using the control PCR product produced, set up the 10µl control ligation reaction

Component	Volume
pCR2.1 TA Cloning vector, (25 ng/µl)	2 µL
5X Ligation Buffer	2 µL
Control PCR Fragment	1 µL
Nuclease-free Water	4 µL
T4 DNA Ligase	1 µL
Total volume	**10 µL**

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the ligation mixture at room temperature (22°C) for 1 hour. Use 1 µL of the ligation mixture directly for bacterial transformation. Keep the ligation mixture at -20°C if transformation is postponed. Thaw on ice and mix carefully before transformation.

   Note. To enable blue/white screening, choose only strains having lacZ∆M15 mutation, for example, XL1-Blue, ER1727, JM109 or other.

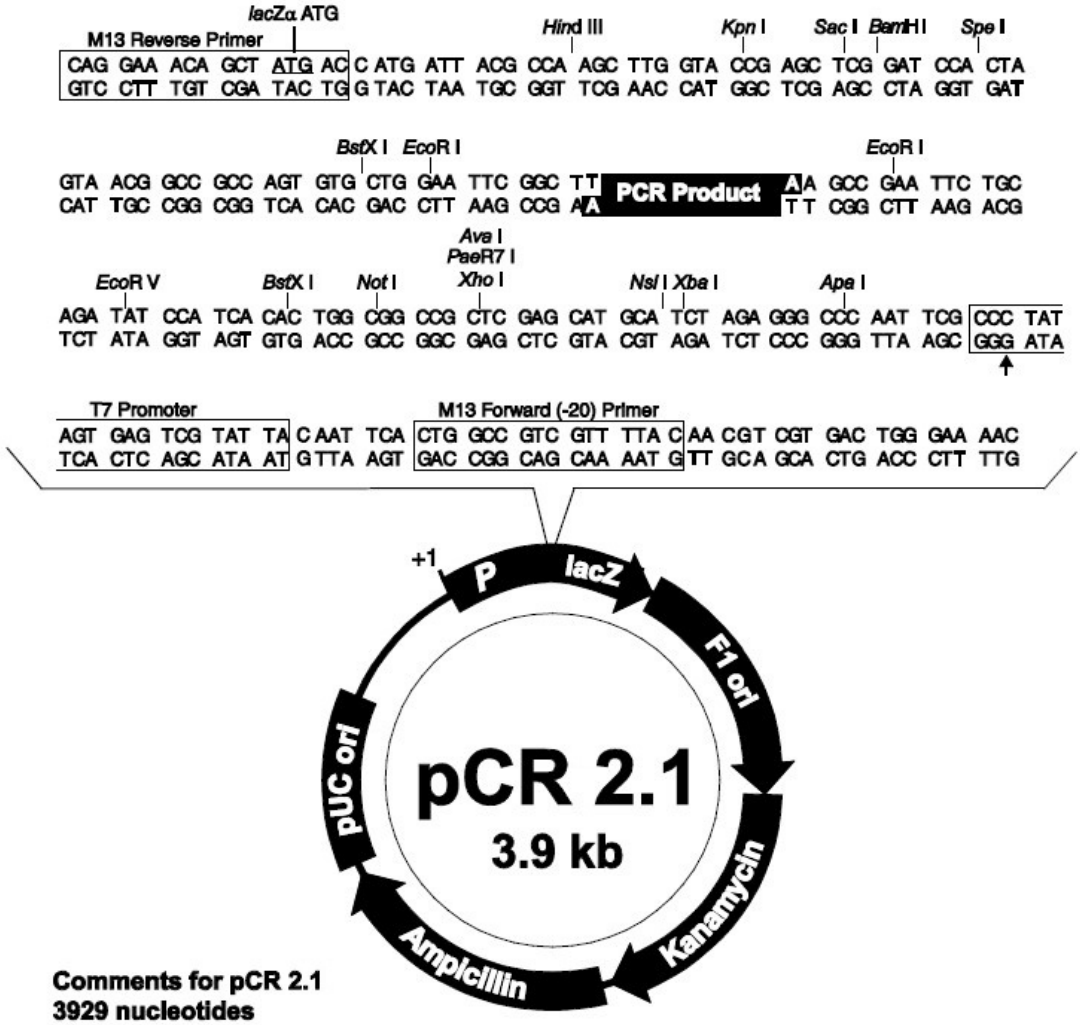
3. Transform 50 µL of competent *E. coli* cells with 1µl of the ligation mixture. Plate the cells on LB amplicillin XGal/IPTG plates.

4. The Control Ligation Reaction should produce approximately 80% white colonies depending on the incubation time and vector to insert ratio. Over time, the 3´ T-overhangs will degrade, causing an increase in the number of background white colonies (those without inserts). The number of background colonies should not exceed 10%. If this occurs, use another vial of pCR2.1 and avoid repeated freeze-thaw cycles.

MAP AND FEATURES OF pCR2.1 CLONING VECTOR

The pCR2.1 TA cloning vector is linearized. The arrow indicates the start of transcription for the T7 RNA polymerase.

The map and the MCS region of the vector are represented below:



Comments for pCR 2.1
3929 nucleotides

LacZα gene: bases 1-545
M13 Reverse priming site: bases 205-221
T7 promoter: bases 362-381
M13 (-20) Forward priming site: bases 389-404
f1 origin: bases 546-983
Kanamycin resistance ORF: bases 1317-2111
Ampicillin resistance ORF: bases 2129-2989
pUC origin: bases 3134-3807

Genetic elements of pCR2.1 TA cloning vector

Feature	Benefit
Lac Promoter	Allows bacterial expression of the lacZα fragment for α-complementation (blue-white screening).
lacZα fragment	Encodes the first 146 amino acids of β-galactosidase. Complementation in trans with the Ω fragment gives active β-galactosidase for blue-white screening.
Kanamycin resistance gene	Allows selection and maintenance in *E. coli*; useful when cloning products amplified from ampicillin-resistant plasmids.
Ampicillin resistance gene	Allows selection and maintenance in *E. coli.*
pUC origin	Allows replication, maintenance, and high copy number in *E. coli.*
T7 promoter and priming site	Allows in vivo or in vitro transcription of anti-sense RNA. Allows sequencing of the insert.
M13 Forward (−20) and M13 Reverse Priming Sites	Allows sequencing of the insert.
f1 origin	Allows rescue of sense strand for mutagenesis and single-strand sequencing.

TROUBLESHOOTING

Problem	Cause and Solution
Few or no transformants	**Low transformation efficiency of competent cells.** Use only high transformation efficiency cells. Perform a control transformation with 0.1 ng of Control PCR fragment (supercoiled pCR2.1 TA DNA). Transformation efficiency should exceed 10^6 cfu/µg DNA. **Proofreading DNA polymerase was used for PCR.** If *Pfu* DNA polymerase, or other proofreading DNA polymerase, was used in PCR, the PCR product is blunt-ended and is not compatible with TA cloning method. Use *Taq* DNA polymerase to generate PCR product for cloning. **T4 DNA Ligase was inhibited by salts present in the PCR buffer.** If using non-purified PCR product, do not add more than 4 µL of the PCR mixture to the ligation reaction to avoid inhibition of T4 DNA ligase by salts. **PCR product was damaged by UV light during excision from the agarose gel.** For efficient cloning of gel-purified DNA fragments, it is important to avoid DNA damage by ethidium bromide and UV light. Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When using a short-wavelength (254-312 nm) light-box, limit DNA exposure to UV to a few seconds. Keep the gel on a glass plate or on a plastic plate during UV illumination. Alternatively, use dyes, like crystal violet, to visualize DNA in ambient light. **Insert:vector ratio is suboptimal.** The optimal insert/vector ratio is 3:1. Refer to Table 1 on page 4 to calculate the amount of PCR product, required for efficient ligation with 0.165 µg (3 µL, 0.172 pmol ends) of the pCR2.1/T vector or use dedicated software for calculations.
Background colonies without plasmid	**Insufficient amount of antibiotic in agar medium.** Use 100 µg/mL of ampicillin in LB-ampicillin agar plates. Allow the LB medium to cool to 55°C before addition of the ampicillin
Background colonies that contain plasmids with incorrect inserts	**PCR products are contaminated with a template which encodes ampicillin resistance.** Gel-purify the PCR product if the PCR template encodes a β-lactamase to avoid background colonies on LB-ampicillin agar. **Non-specific PCR products or primer dimers were cloned.** Gel-analyze the PCR product prior to ligation. If non-specific PCR products or primer-dimers were generated during

Problem	Cause and Solution
Background colonies that contain plasmids with incorrect inserts	**Large PCR product (>1 kb) was cloned without purification.** Short DNA fragments (<1 kb) are cloned with a much higher efficiency compared to long ones. Therefore, long PCR products must be purified to remove any smaller fragments from the solution. **Nuclease contamination.** To guaranty DNA integrity, preserve long PCR product from both mechanical sharing and damage by nucleases: • Use only components provided with the kit. Nuclease contamination can impair the integrity of the cloning vector, thus disabling blue/white selection of recombinant clones. • Store PCR product at -20 °C if it is not used immediately. Use clean labware and razor blade, prepare fresh electrophoresis running buffer for gel purification procedure.
False negatives in colony PCR	**False-negatives in colony PCR.** Due to considerable amount of recircularized vector plated on the surface of the plate, colony PCR may give some false-negative results. Prior to clone analysis propagate short strikes of individual colonies on ampicillin plates. Then use small amount of each for colony PCR.
Transformation efficiency is too low	**Competent cells prepared from non-fresh bacterial culture.** Seed overnight culture from a freshly streaked bacterial culture plate. Refresh bacterial strains weekly. For seeding of overnight *E. coli* DH5α culture, use only one day old culture plates.
Sequence errors in the cloned insert	**PCR product was damaged by UV light during excision from agarose gel.** Use a long wavelength UV (360 nm) light-box when excising DNA from the agarose gel. When a short-wavelength (254-312 nm) light-box is used, limit DNA exposure to UV to a few seconds. Keep the gel on a glass or on plastic plate during UV illumination. Alternatively, use dyes visible in ambient light to visualize DNA in standard agarose gels. **Errors in PCR primers.** If the cloned PCR product contains sequence errors or is missing 5' bases and the same error persists in more than one clone, re-order the PCR primers from a reliable supplier and repeat the procedure starting from the PCR step.

RECIPES

Ampicillin stock solution (50 mg/mL)

Dissolve 2.5 g of ampicillin sodium salt in 50 mL of deionized water. Filter-sterilize and store in aliquots at -20 °C.

X-Gal stock solution (20 mg/mL)

Dissolve 200 mg X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) in 10 mL N,N-dimethylformamide. Store at -20 °C in the dark. Alternatively, use X-Gal Solution, ready-to-use. Use 40 µL per plate.

IPTG stock solution (100 mM)

Dissolve 1.2 g IPTG (isopropyl- β -D-thiogalactopyranoside) in 50 mL deionized water. Filter-sterilize, aliquote and store at 4 °C. Alternatively, use IPTG Solution, ready-to-use. Use 40 µL per plate.

LB-ampicillin X-Gal/IPTG Plates

- Prepare LB-agar medium (1 liter), weigh out:

Tryptone	10 g,
Yeast extract	5 g,
NaCl	10 g.

  Dissolve in 800 mL of water, adjust pH to 7.0 with NaOH and adjust the volume with water to 1000 mL. Add 15 g of agar and autoclave.

- Before pouring the plates, allow the medium to cool to 55 °C. Then, add 1 mL of ampicillin stock solution (50 mg/mL) to a final concentration of 50 µg/mL. Mix gently and pour the plates. Allow the LB-ampicillin agar medium to solidify. Dry plates opened at room temperature under UV light for 30 min.
- Add 40 µL of X-Gal stock solution (20 mg/mL) or X-Gal Solution, ready-to-use and 40 µL of IPTG 100 mM or IPTG Solution, ready-to-use, spread evenly with a sterile spatula.