Thermo SCIENTIFIC

PRODUCT INFORMATION

Thermo Scientific CloneJET PCR Cloning Kit

#K1231 **20 rxns**

Lot **Expiry Date**

Store at -20 °C

www.thermoscientific.com/onebio

COMPONENTS OF THE KIT

CloneJET PCR Cloning Kit	#K1231 20 cloning rxns	#K1232 40 cloning rxns
pJET1.2/blunt Cloning Vector (50 ng/µL)	24 µL	46 µL
2X Reaction Buffer	300 µL	600 µL
T4 DNA Ligase (5 U/µL)	24 µL	46 µL
DNA Blunting Enzyme	24 µL	46 µL
pJET1.2 Forward Sequencing Primer, 10 µM aqueous solution	50 µL	100 µL
pJET1.2 Reverse Sequencing Primer, 10 µM aqueous solution	50 µL	100 µL
Control PCR Product (24 ng/µL) 976 bp, with 3'-dA overhangs	8 µL	12 µL
Water, nuclease-free	1.25 mL	1.25 mL

STORAGE

All components of the Thermo Scientific™ CloneJET™ PCR Cloning Kit should be stored at -20°C.

CERTIFICATE OF ANALYSIS

All components of the kit were function tested in control experiment as described in the manual.
A 2 µL aliquot of the ligation mixture was used to transform 50 µL of chemically competent XL1-Blue cells of >10^6 cfu/µg DNA transformation efficiency.
Cloning efficiency of the Control PCR Product into the pJET1.2/blunt was >2x10^4 cfu/µg DNA. >90% of the recombinant plasmids contained the appropriate insert.

Quality authorized by: Jurgita Zilinskiene

DESCRIPTION

The CloneJET PCR Cloning Kit is an advanced positive selection system for the highest efficiency cloning of PCR products generated with *Pfu* DNA polymerase, *Taq* DNA polymerase, Thermo Scientific™ DreamTaq™ DNA polymerase or other thermostable DNA polymerases. Additionally, any other DNA fragment, either blunt or sticky-end, can be successfully cloned using the kit.
The kit features the novel positive selection cloning vector pJET1.2/blunt. This vector contains a lethal gene which is disrupted by ligation of a DNA insert into the cloning site. As a result, only cells with recombinant plasmids are able to propagate, eliminating the need for expensive blue/white screening.
The vector contains an expanded multiple cloning site, as well as a T7 promoter for *in vitro* transcription. Sequencing primers are included for convenient sequencing of the cloned insert.

CLONING PRINCIPLE

pJET1.2/blunt is a linearized cloning vector, which accepts inserts from 6 bp to 10 kb. The 5'-ends of the vector contain phosphoryl groups, therefore, phosphorylation of the PCR primers is not required.
Blunt-end PCR products generated by proofreading DNA polymerases can be directly ligated with the vector in just 5 min. PCR products with 3'-dA overhangs generated using *Taq* DNA polymerase or other non-proofreading thermostable DNA polymerase are blunted in 5 min with a proprietary thermostable DNA blunting enzyme (included in the kit) prior to ligation. All common laboratory *E. coli* strains can be directly transformed with the ligation product.

IMPORTANT NOTES

- Thoroughly mix every vial before use.
- The CloneJET PCR Cloning Kit is compatible with all PCR buffers supplied by Thermo Scientific.
- 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 1 µL of unpurified PCR product in the blunting or ligation reaction. Excess polymerase (*Taq*, *Pfu* or other) or salts from the PCR reaction mixture may result in background colonies and may reduce the efficiency of the cloning procedure.
- Gel purification of the PCR product (e.g. with Thermo Scientific™ GeneJET™ Gel Extraction Kit, #K0691) 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 (1, 2).
- The kit performs well over a wide range of insert/vector molar ratios (0.5:1 to 15:1). However the optimal insert/vector ratio is 3:1. Vector pJET1.2/blunt is supplied at a concentration of 0.05 pmol DNA ends/µL. To calculate optimal amount of the PCR product for ligation (0.15 pmol of DNA ends respectively), refer to Table 1 or use dedicated software (e.g., www.thermoscientific.com/reviewer) for calculations.
- For PCR products >3 kb, ligation can be prolonged to 30 min. Ligation times longer than 30 min are not recommended and may decrease cloning efficiency.
- Success in cloning of long PCR products may also depend on the DNA sequence of the insert. PCR products may contain toxic sequences not tolerated by *E.coli*, therefore multicopy vectors like pJET1.2 may not be suitable cloning these PCR products.

Table 1. Recommended amount of PCR product for the ligation reaction

Length of PCR product (bp)	Optimal PCR product quantity for ligation reaction, (0.15 pmol ends)
100	5 ng
300	15 ng
500	25 ng
1000	50 ng
2000	100 ng
3000	150 ng
4000	200 ng
5000	250 ng

CLONING PROTOCOLS

Blunt-End Cloning Protocol

- For cloning blunt-end PCR products generated by proofreading DNA polymerases, such as *Pfu* DNA polymerase. If the DNA end structure of the PCR products is not specified by the supplier of the DNA polymerase, follow the Sticky-End Cloning Protocol.
- For cloning of blunt-end DNA fragments generated by restriction enzyme digestion. Gel-purify the DNA fragment prior to ligation and use in a 3:1 molar ratio with pJET1.2/blunt (see Table 1).

1. Set up the ligation reaction **on ice**:

Component	Volume
2X Reaction Buffer	10 µL
Non-purified PCR product *or* purified PCR product/other blunt-end DNA fragment	1 µL 0.15 pmol ends
pJET1.2/blunt Cloning Vector (50 ng/µL)	1 µL (0.05 pmol ends)
Water, nuclease-free	up to 19 µL
T4 DNA Ligase	1 µL
Total volume	20 µL

Vortex briefly and centrifuge for 3-5 s.
2. Incubate the ligation mixture at room temperature (22°C) for 5 min.
Note. *For PCR products >3 kb, ligation can be prolonged to 30 min.*
3. Use the ligation mixture directly for transformation
Note. *Keep the ligation mixture at -20°C if transformation is postponed. Thaw on ice and mix carefully before transformation.*

Sticky-End Cloning Protocol

- For cloning PCR products with 3'-dA overhangs generated by *Taq* DNA polymerase, DreamTaq DNA polymerase or enzyme mixtures containing *Taq* DNA polymerase.
- For cloning PCR products when DNA end structure of the generated PCR products is not specified by the supplier of the DNA polymerase.
- For cloning DNA fragments with 5'- or 3'-overhangs generated by restriction enzyme digestion. Gel-purify the DNA fragment prior to ligation and use in a 3:1 molar ratio with pJET1.2/blunt (*see* Table1).
Note. *The DNA Blunting Enzyme is a proprietary thermostable DNA polymerase with proofreading activity. It will remove 3'- overhangs and fill-in 5'-overhangs. Nucleotides for the blunting reaction are included in the reaction buffer.*

1.Set up the blunting reaction **on ice**:

Component	Volume
2X Reaction Buffer	10 µL
Non-purified PCR product *or* purified PCR product/other sticky-end DNA fragment	1 µL (0.15 pmol ends)
Water, nuclease-free	to 17 µL
DNA Blunting Enzyme	1 µL
Total volume	18 µL

Vortex briefly and centrifuge for 3-5 s.
2. Incubate the mixture at 70 °C for 5 min. Chill on ice.
3. Set up the ligation reaction **on ice**. Add the following to the blunting reaction mixture:

Component	Volume
pJET1.2/blunt Cloning Vector (50 ng/µL)	1 µL (0.05 pmol ends)
T4 DNA Ligase	1 µL
Total volume	20 µL

Vortex briefly and centrifuge for 3-5 s to collect drops.
4. Incubate the ligation mixture at room temperature (22 °C) for 5 min.
Note. *For PCR products >3 kb, ligation can be prolonged to 30 min.*
5. Use the ligation mixture directly for transformation
Note. *Keep the ligation mixture at -20 ˚C if transformation is postponed. Thaw on ice and mix carefully before transformation.*

Transformation

- The CloneJET PCR Cloning Kit is compatible with all common *E.coli* laboratory strains. Transformation of competent *E.coli* cells with the ligation mixture can be performed using different transformation methods (Table 2 and 3).
- The number of transformants on the plates directly depends on the transformation efficiency of the competent cells.
- For successful cloning, competent *E.coli* cells should have an efficiency of at least 1 × 10^6 cfu/ µg supercoiled plasmid DNA. To check the efficiency, prepare a control transformation with 0.1 ng of a supercoiled vector DNA, e.g., pUC19 DNA, #SD0061 (Table 2).
- For transformation of the ligation mixture, refer to Table 3.

Table 2. Evaluation of transformation efficiency of competent cells (control transformation)

Transformation method	Number of transformants per µg of supercoiled plasmid DNA	Amount of pUC19 DNA to yield ~ 1000 colonies per plate	Volume of competent cells
Thermo Scientific™ TransformAid™ Bacterial Transformation Kit, #K2710*	~1 × 10^7	0.1 ng	50 µL
Calcium chloride transformation	~1 × 10^6	1 ng	50 µL
Electro-transformation	~1 × 10^9	0.01 ng	40 µL

***** XL1-Blue, ER2267, ER1727 *E. coli* strains are the best strains for transformation with TransformAid™ Bacterial Transformation Kit. DH10B, DH5α and TOP10 strains are not efficient with TransformAid but are recommended for calcium chloride transformation or electroporation.

Table 3. Recommendations for transformation of ligation mixture

Tranformation method	Treatment of the ligation mixture before transformation	Volume of the ligation mixture for transformation	Volume of competent cells for transforma-tion
TransformAid Bacterial Transformation Kit, #K2710	Do not heat ligation mixture prior used for transformation.	≤2.5 µL	50 µL
Calcium Chloride Transformation	Do not heat ligation mixture prior used for transformation.	≤5 µL	50 µL
Electro-transformation	Spin column or chloroform extraction (*see* protocol below) Do not heat ligation mixture prior used for transformation.	0.5-1 µL of purified ligation mixture	40 µL

Analysis of recombinant clones

Analyze 4-6 colonies for the presence and orientation of the DNA insert using one of the following methods:

Colony PCR

Use the following protocol for colony screening by PCR if the cloned PCR fragment is shorter than 3 kb. For longer inserts, perform restriction analysis.

1. Prepare enough PCR master mix for the number of colonies analyzed plus one extra. For each 20 µL reaction, mix the following reagents:

Component	Using *Taq* DNA Polymerase	Using 2X PCR Master Mix
10X *Taq* buffer	2.0 µL	–
dNTP mix, 2 mM each	2.0 µL	–
25 mM $MgCl_2$	1.2 µL	–
pJET1.2 Forward Sequencing Primer, 10 µM	0.4 µL	0.4 µL
pJET1.2 Reverse Sequencing Primer, 10 µM	0.4 µL	0.4 µL
Water, nuclease-free	13.9 µL	9.2 µL
Taq DNA Polymerase 5 u/µL, #EP0401 *or* DreamTaq Green DNA Polymerase, #EP0711	0.1 µL	–
PCR Master Mix (2X), #K0171 *or* DreamTaq Green PCR Master Mix (2X), #K1081	–	10 µL
Total volume	20 µL	20 µL

2. Mix well. Aliquot 20 µL of the mix into the PCR tubes on ice.
3. Pick a small amount of individual colony and resuspend in 20 µL of the PCR master mix. *Picking a colony, take care not to touch the agar.*

Note. *Due to considerable amount of recircularised vector plated on the surface of 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.*

4. Perform PCR: 95 °C, 3 min; 94 °C, 30 s, 60 °C, 30 s, 72 °C 1 min/kb; 25 cycles.
5. Analyze on an agarose gel for the presence of the PCR product.

Restriction analysis

Isolate plasmid DNA from an overnight bacterial culture. To speed up the process and to assure the quality of purified plasmid DNA, use the Thermo Scientific™ GeneJET™ Plasmid Miniprep Kit, #K0503. To digest DNA from recombinant clones in just 5 min, use Thermo Scientific™ FastDigest™ restriction enzymes.

Sequencing

Use the pJET1.2 Forward Sequencing Primer or pJET1.2 Reverse Sequencing Primer supplied with the kit to sequence the cloned insert.

CONTROL CLONING EXPERIMENT

The control reaction should be used to verify the efficiency of the blunting and ligation steps. The 976 bp control PCR product (nucleotide sequence is available at www.thermoscientific.com/onebio) has been generated with *Taq* DNA polymerase, which adds extra nucleotides to the 3'-end. Therefore, the **Sticky-End Protocol** must be followed.

1. Set up the blunting reaction **on ice**:

Component	Volume
2X Reaction Buffer	10 µL
Control PCR Product (24 ng/µL)	2 µL
Water, nuclease-free	5 µL
DNA Blunting Enzyme	1 µL
Total volume	18 µL

   Vortex briefly and centrifuge for 3-5 s to collect drops.
2. Incubate the mixture at 70 °C for 5 min. Chill on ice.
3. Set up the ligation reaction **on ice**. Add the following to the blunting reaction mixture:

Component	Volume
pJET1.2/blunt Cloning Vector (50 ng/µL)	1 µL
T4 DNA Ligase	1 µL
Total volume	20 µL

   Vortex briefly and centrifuge for 3-5 s to collect drops.
4. Incubate the ligation mixture at room temperature (22 °C) for 5 min.
5. Use the ligation mixture directly for transformation. Keep the ligation mixture at -20°C if transformation is postponed. Thaw on ice and mix carefully before transformation.
   Analyze colonies by colony PCR. At least 9 of 10 analyzed colonies should contain recombinant plasmid with the 976 bp insert.
   The number of transformants depends on the transformation efficiency of the *E. coli* cells. Verify the transformation efficiency by transforming supercoiled plasmid, e.g., pUC19 DNA, #SD0061 in parallel. Refer to Table 2 for correct control transformations.

MAP AND FEATURES OF pJET1.2/blunt CLONING VECTOR

The pJET1.2/blunt cloning vector has been linearized with Eco32I (EcoRV) (GenBank/EMBL Accession number EF694056). The blunt ends of the vector contain 5'-phosphoryl groups. The nucleotide sequence of pJET1.2/blunt is available at www.thermoscientific.com/onebio. For complete list of enzymes that cut or do not cut the pJET1.2/blunt *see* www.thermoscientific.com/reviewer.

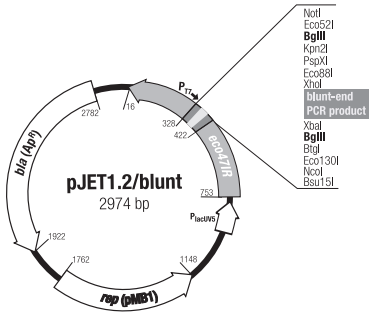

Fig. 1. pJET1.2/blunt Vector Map.

Genetic elements of the pJET1.2/blunt cloning vector

Element	Function	Position (bp)
rep (pMB1)	Replicon (rep) from the pMBI plasmid responsible for the replication of pJET1.2	1762-1148
Replication start	Initiation of replication	1162±1
bla (Ap^R)	β-lactamase gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells	2782-1922
eco47IR	Lethal gene *eco47IR* enables positive selection of recombinant plasmid	753-16
P_{lacUV5}	Modified P_{lac} promoter for expression of the *eco47IR* gene at a level sufficient to allow for positive selection	892-769
T7 promoter	T7 RNA polymerase promoter for *in vitro* transcription of the cloned insert	305-324
Multiple cloning site (MCS)	Mapping, screening and excision of the cloned insert	422-328
Insertion site	Blunt DNA ends for ligation with insert	371-372
Primer binding sites:		
pJET1.2 forward sequencing primer	Sequencing of insert, colony PCR. Sequence: 5'-CGACTCACTATAGGGAGAGCGGC-3'	310-332
pJET1.2 reverse sequencing primer	Sequencing of insert, colony PCR. Sequence: 5'-AAGAACATCGATTTTCCATGGCAG-3'	428-405

RECIPES & SUPPLEMENTARY PROTOCOLS

Chloroform extraction of the ligation mixture prior electroporation

1. Add an equal volume (20 µL) of the chloroform to the ligation mixture. Mix well.
2. Centrifuge at 10,000 rpm for 3 min at room temperature.
3. Carefully transfer the upper aqueous phase to a fresh tube.
4. Use 1 µL of the purified mixture for transformation of 40 µL of electrocompetent cells.

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

Ampicillin stock solution (50 mg/mL)

1. Dissolve 2.5 g ampicillin sodium salt in 50 mL of deionized water.
2. Filter sterilize and store in aliquots at 4 °C.

LB-ampicillin plates

1. Prepare LB-agar Medium (1 liter), weigh out:
   Bacto® Tryptone 10 g,
   Bacto Yeast extract 5 g,
   NaCl 5 g.
2. Dissolve in 800 mL of water, adjust pH to 7.0 with NaOH and add water to 1000 mL.
3. Add 15 g of agar and autoclave.
4. Allow the medium to cool to 55 °C.
5. Add 2 mL of ampicillin stock solution (50 mg/mL) to a final concentration of 100 µg/mL.
6. Mix gently and pour plates.

TROUBLESHOOTING

For troubleshooting *see* the complete version of product information for CloneJET PCR Cloning Kit at www.thermoscientific.com/onebio.

References

1. Rand, K.N., Crystal Violet can be used to Visualize DNA Bands during Gel Electrophoresis and to Improve Cloning Efficiency, Elsevier Trends Journals Technical Tips, Online, T40022, 1996.
2. Adkins, S., Burmeister, M., Visualization of DNA in agarose gels and educational demonstrations, Anal Biochem., 240 (1), 17-23, 1996.