CloneJET™ PCR Cloning Kit
#K1231, #K1232

CERTIFICATE OF ANALYSIS

CloneJET™ PCR Cloning Kit

#___
Lot __

QUALITY CONTROL

This lot of the kit has been tested for cloning of the 976 bp control PCR product. A 2 µl aliquot of the ligation mixture was used to transform 50 µl of chemically competent XL1-Blue cells. Transformation efficiency of the cells with pUC19 was 0.19×10^7 cfu/µg. Cloning efficiency of the Control PCR Product into the pJET1.2/blunt exceeded 0.5×10^5 cfu/µg. 99% of the recombinant plasmids contained the appropriate insert.

Quality authorized by:  Jurgita Zilinskiene

CONTENTS page

COMPONENTS OF THE KIT

CloneJET™ PCR Cloning Kit	**20 cloning reactions** #K1231	**40 cloning reactions** #K1232
pJET1.2/blunt Cloning Vector (50 ng/µl)	24 µl	46 µl
2X Reaction Buffer	240 µl	460 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, 10µM 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 CloneJET™ PCR Cloning Kit should be stored at -20°C.

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, 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. Cloning is fast and efficient; ligation takes only 5 minutes and yields more than 99% positive clones.

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 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 cloning site 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 in just 5 min with the pJET1.2/blunt cloning vector. PCR products with 3'-dA overhangs generated using *Taq* DNA polymerase or other non-proofreading thermostable DNA polymerases 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.

Recircularized pJET1.2/blunt vector expresses a lethal restriction enzyme after transformation and is not propagated. As a result, only recombinant clones containing the insert appear on culture plates. Therefore, blue/white screening is not required.

IMPORTANT NOTES

- DEPC-treat all tubes and pipette tips to be used in cDNA synthesis or use certified nuclease-free labware.
- Thoroughly mix every vial before use.
- Gel-analyze the PCR product for specificity and yield before cloning.
- Purification of the PCR product is generally not required, unless:
  – PCR product is contaminated with non-specific PCR products;
  – PCR product is contaminated with primer-dimers;
  – PCR product is longer than 1kb;
  – PCR template contains b-lactamase (ampicillin resistance) gene, which may result in background colonies on LB-ampicillin agar plates.
- For efficient cloning of gel-purified DNA fragments, care should be taken to avoid DNA damage with UV light. Keep the gel on a glass or plastic plate during UV illumination. Minimize UV exposure to a few seconds. To avoid DNA exposure to UV altogether, visible dyes can be included in standard agarose gels to visualize DNA bands in ambient light (1, 2).
- The CloneJET™ PCR Cloning Kit is compatible with all PCR buffers supplied by Fermentas.
- The kit performs well over a wide range of insert/vector molar ratios (0.5:1 to 15:1). The optimal insert/vector ratio is 3:1.

Blunt-End Cloning Protocol

- Use for cloning blunt-end PCR products generated by proofreading DNA polymerases, such as *Pfu* DNA polymerase.
- If the end structure of the PCR products is not specified by the supplier of the DNA polymerase, follow the Sticky-End Cloning Protocol.
- This protocol can also be used for cloning blunt-end DNA fragments generated by restriction digestion. Gel-purify the DNA fragment prior to ligation and use in a 3:1 molar ratio with pJET1.2/blunt. *See* www.fermentas.com/reviewer for molar conversions.

1. Set up the ligation reaction:

Component	Volume
2X Reaction Buffer	10 µl
PCR product	1-2 µl*
pJET1.2/blunt Cloning Vector (50 ng/µl)	1 µl
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. Incubation time can be extended up to 30 min if the maximal number of transformants is required.

3. Use the ligation mixture directly for bacterial transformation (*see* p.6).

*If using non-purified PCR product, do not exceed 2 µl of the PCR mixture to avoid inhibition of T4 DNA ligase by salts present in the PCR buffer. Purification of the PCR fragment prior to cloning is recommended in cases described in p.3. Purified PCR product is used at a 3:1 molar ratio with the cloning vector.

Sticky-End Cloning Protocol

- Use for cloning PCR products with 3'-dA overhangs generated by *Taq* DNA polymerase, DreamTaq™ DNA polymerase or enzyme mixtures containing *Taq* DNA polymerase.
- If the end structure of the PCR products is not specified by the supplier of the DNA polymerase, follow the Sticky-End Cloning Protocol.
- This protocol can also be used for cloning DNA fragments with 5‘- or 3‘-overhangs generated by restriction digestion. Gel-purify the DNA fragment prior to ligation and use in a 3:1 molar ratio with pJET1.2/blunt. *See* www.fermentas.com/reviewer for molar conversions.
- The DNA blunting enzyme is a proprietary thermostabile DNA polymerase with proofreading activity. It will remove 3'- overhangs and fill-in 5'-overhangs. Nucleotides for the blunting reaction are supplied in the reaction buffer.

1. Set up the blunting reaction:

Component	Volume
2X Reaction Buffer	10 µl
PCR product	1-2 µl*
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 briefly on ice.

3. Set up the ligation reaction. 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.

4. Incubate the ligation mixture at room temperature (22°C) for 5 min.

Note. Incubation time can be extended up to 30 min if the maximal number of transformants is required.

5. Use the ligation mixture directly for bacterial transformation (*see* p.6).

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*If using non-purified PCR product, do not exceed 2 µl of the PCR mixture to avoid inhibition of T4 DNA ligase by salts present in the PCR buffer. Purification of the PCR fragment prior to cloning is recommended in cases described in p.3. Purified PCR product is used at a 3:1 molar ratio with the cloning vector.

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 a number of different transformation methods. Use competent *E.coli* cells with a transformation efficiency of at least $1x10^6$ transformants per µg of supercoil plasmid DNA.

Transformation tips

- For fast cloning, we recommend using competent cells prepared with the TransformAid™ Bacterial Transformation Kit (#K2710). Use up to 2.5 µl of the ligation reaction mixture to transform 50 µl of competent *E. coli* cells prepared with this kit.
- To transform competent *E. coli* cells prepared by the calcium chloride method, use up to 5 µl of the ligation mixture per 50 µl of competent cells.
- For transformation by electroporation, extract the ligation reaction mixture with chloroform. Use 1 µl of the extracted ligation mixture to transform 50 µl of competent cells.
- The highest efficiency in transformation of the ligation mixture was observed with the following *E.coli* strains: XL1-Blue, ER2267, ER1727, DH10B, TOP10.

Transformation of competent E.coli cells prepared with TransformAid™ Bacterial Transformation Kit (#K2710)

1. Prepare LB-ampicillin agar plates (*see* p.11). Pre-warm the plates at 37°C for at least 20 min.
2. Prepare competent *E.coli* cells as described in the protocol provided with the TransformAid™ Bacterial Transformation Kit.
3. Transfer 2.5 µl of the ligation mixture into a new microcentrifuge tube. Chill 2 min on ice.
4. Add 50 µl of the prepared competent *E.coli* cells. Incubate on ice for 5 min.
5. Plate immediately on pre-warmed LB-ampicillin agar plates. Incubate overnight at 37°C.

Analysis of recombinant clones

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

- **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 GeneJET™ Plasmid Miniprep Kit (#K0503). To digest DNA from recombinant clones in just 5 minutes, use 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.
- **Colony PCR**
  Use the following protocol for colony screening by PCR.

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** (#EP0402)	**Using 2X PCR Master Mix** (#K0171)
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	0.1 µl	–
2X PCR master mix	–	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 an individual colony and resuspend in 20 µl of the PCR master mix.
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.

CONTROL 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.fermentas.com) 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:

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.

2. Incubate the mixture at 70°C for 5 min. Chill on ice for several seconds.
3. Follow with the ligation reaction. Add 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.

4. Incubate the ligation mixture at room temperature (22°C) for 5 min.
5. Transform chemically competent *E.coli* cells with 2.5 µl of the ligation mixture, or electrocompetent cells with 1 µl of chloroform-extracted ligation mixture.

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 50 µl of cells with 0.1 ng of a supercoiled circular plasmid, e.g. pUC19 DNA (#SD0061). The transformation efficiency should be at least $1x10^6$ cfu/µg DNA.

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.fermentas.com.

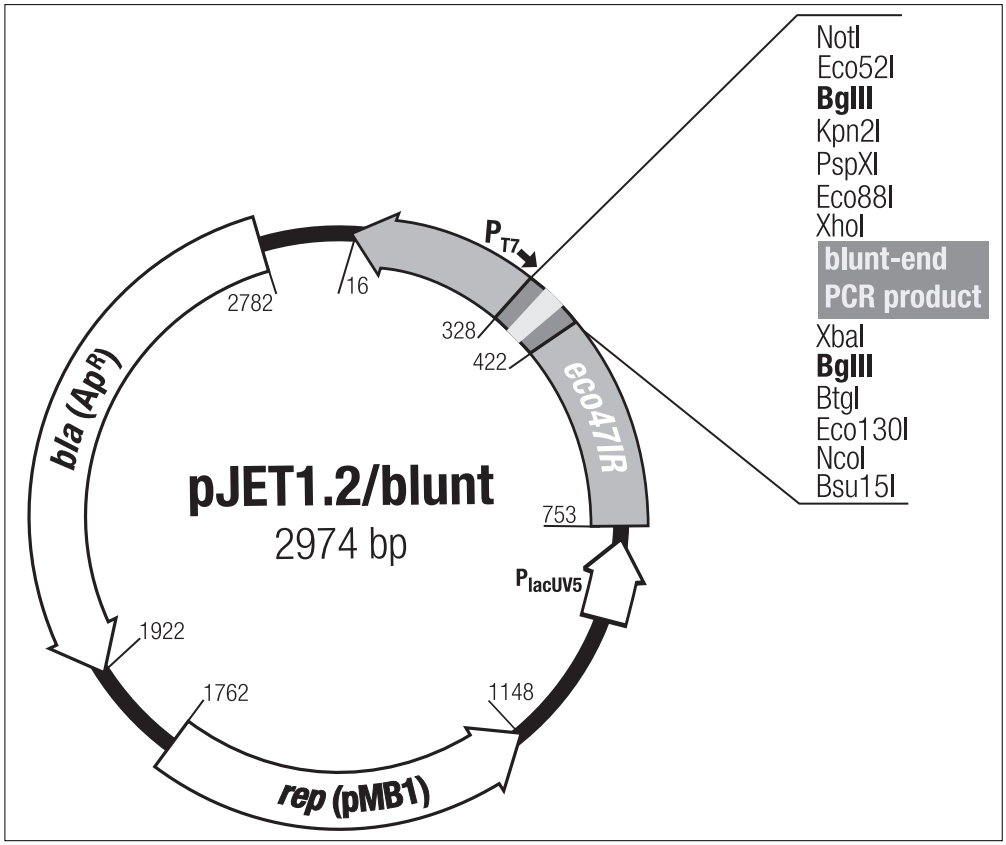

Fig. 1. pJET1.2/blunt Vector Map.

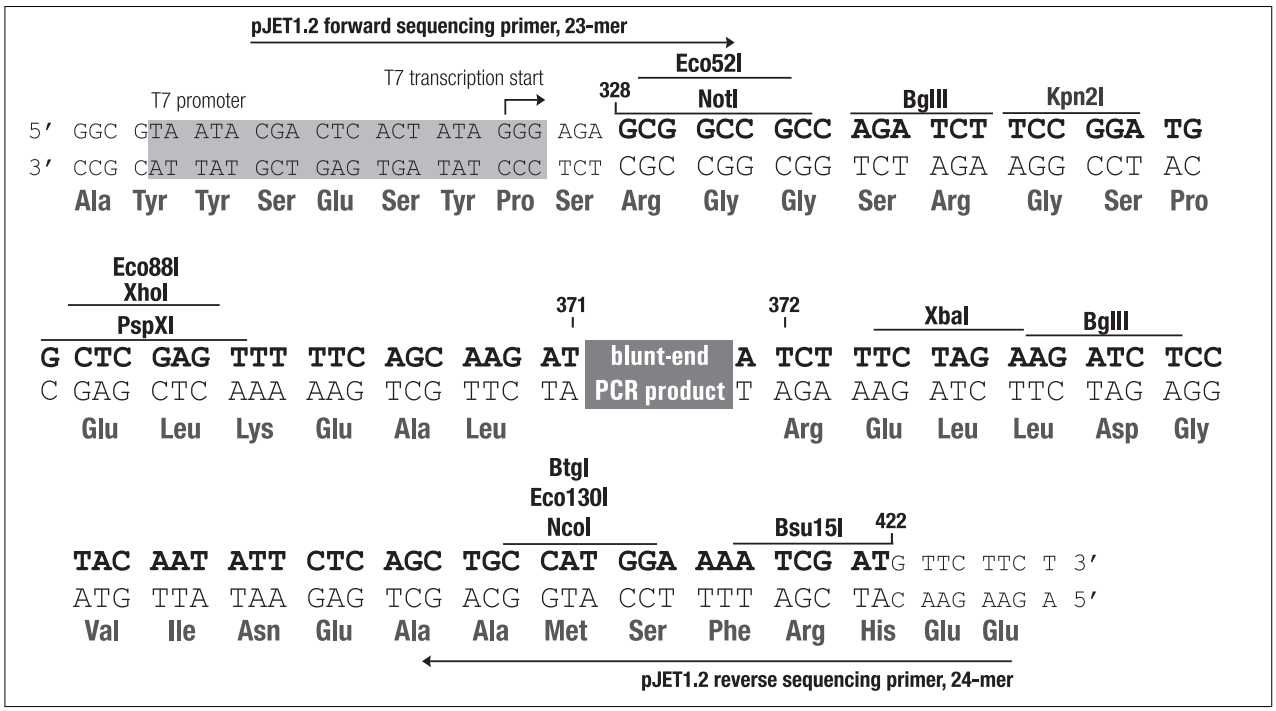

Fig. 2. DNA Sequence of MCS region.

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	310-332
pJET1.2 reverse sequencing primer	Sequencing of insert, colony PCR	428-405

Primer sequences

pJET1.2 forward sequencing primer, 23-mer	5'-CGACTCACTATAGGGAGAGCGGC-3'
pJET1.2 reverse sequencing primer, 24-mer	5'-AAGAACATCGATTTTCCATGGCAG-3'

Fermentas restriction enzymes that do not cut pJET1.2/blunt

For complete list of enzymes *see* www.fermentas.com/reviewer.

AarI, Acc65I, AjiI, AjuI, AlfI, ApaI, BamHI, BclI, BcuI , BoxI , BplI, Bpu1102I , BseJI, BshTI, Bsp119I, Bsp120I, Bsp1407I, Bsp68I, BspTI, Bst1107I, BstXI, Cfr42I, Cfr9I, CpoI, Ecl136II, Eco105I, Eco147I, Eco24I, Eco32I, Eco47III, Eco72I, Eco81I, Eco91I, EcoO109I, EcoRI, EheI, FspAI, HincII, KpnI, KspAI, MlsI, MluI, Mph1103I, NdeI, NheI, OliI, PaeI, PasI, PauI, PdiI, Pfl23II, PfoI, Ppu21I, Psp5II, PsyI, SacI, SalI, SdaI, SfiI, SgsI, SmaI, TstI, Van91I, XagI, XmaJI, XmiI.

Fermentas restriction enzymes that cut pJET1.2/blunt once

For complete list of enzymes *see* www.fermentas.com/reviewer.

Enzyme	Location
AasI	1204
AdeI	120
AloI	284
BfiI	2040
BglI	2109
BpiI	58
Bpu10I	717
Bsu15I	417
BveI	219
CaiI	1513
Cfr10I	2075
Csp6I	2474
Eam1105I	1990

Enzyme	Location
Eco130I	408
Eco31I	2062
Eco52I	329
Eco88I	352
Esp3I	739
FaqI	103
GsuI	2080
HindIII	624
Kpn2I	343
LguI	979
MssI	761
MunI	892
Mva1269I	722

Enzyme	Location
NcoI	408
NotI	328
NsbI	2215
PdmI	2590
PstI	5
PvuI	2362
RsaI	2474
ScaI	2473
SmuI	951
TatI	2473
XbaI	377
XhoI	352

RECIPES

Ampicillin stock solution (50 mg/ml)

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

LB-ampicillin plates

Prepare LB-agar Medium (1 liter), weigh out:
Bacto Tryptone® 10 g,
Bacto Yeast extract® 5 g,
NaCl 5 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 LB-ampicillin agar plates, allow the medium to cool to 55°C. Add 2 ml of ampicillin stock solution (50 mg/ml) to a final concentration of 100 µg/ml. Mix gently and pour plates.

For fast and easy preparation of LB medium and LB agar plates supplemented with ampicillin, use pre-mixed and pre-sterilized microwaveable FastMedia™ LB Liquid Amp (#M0011) and FastMedia™ LB Agar Amp (#M0021).

TROUBLESHOOTING

Problem	Cause and Solution
Few or no transformants	**Low transformation efficiency of competent *E. coli* cells.** Verify transformation efficiency with 0.1 ng of a supercoiled vector DNA (e.g., pUC19). The competent cells should yield at least $1x10^6$ transformants per µg of supercoiled DNA. **Ligase was not removed prior to electroporation.** Ensure that the ligation reaction mixture was extracted with chloroform prior to electroporation. **Incorrect protocol was used.** If *Taq* DNA polymerase or any enzyme mix containing *Taq* DNA polymerase was used for PCR, always follow the Sticky-End Protocol to blunt the PCR product prior to ligation. **T4 DNA Ligase was inhibited by salts present in the PCR buffer.** Do not use more than 2.0 µl of the PCR mixture in the ligation reaction to avoid inhibition of T4 DNA ligase by salts, or purify PCR product prior to cloning. **PCR product was damaged by UV light during excision from the agarose gel.** 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 visible in ambient light to visualize DNA in standard agarose gels (1, 2). **Cloned sequence is not tolerated by *E.coli*.** Check the target sequence for strong *E.coli* promoters or other potentially toxic elements, as well as inverted repeats. In cases where the product of a cloned gene is toxic to the host, use promoters with a very low expression background or choose a low copy plasmid as cloning vehicle.
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.

Problem	Cause and Solution
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 into pJET1.2/blunt.** Gel-analyze the PCR product prior ligation with the pJET1.2/blunt. If non-specific PCR products or primer-dimers were generated during the PCR reaction, gel-purify the target PCR product. Otherwise, optimize the PCR conditions to increase specificity.
Background colonies that contain plasmids without inserts	**Nuclease contamination.** Use only components provided with the kit. Nuclease contamination (e.g. from low quality water) can impair the integrity of the lethal gene, thus disabling positive selection with pJET1.2/blunt. **False-negatives in colony PCR.** 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.
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 (1, 2). **Low fidelity DNA polymerase was used in PCR.** If PCR product will be used for cloning it is always recommended to use high fidelity DNA polymerase with proofreading activity, such as *Pfu* DNA Polymerase (#EP0671) or High Fidelity PCR Enzyme Mix (#K0191). **Errors in PCR primers.** If the cloned PCR product contains sequence errors or is missing 5' bases and the same error persits in more than one clone, re-order the PCR primers from a reliable supplier and repeat the procedure starting from the PCR step.

QUALITY CONTROL

Each lot of the kit has been tested for efficient cloning of the 976 bp Control PCR Product. A 2 µl aliquot of the ligation mixture was used to transform 50 µl of chemically competent XL1-Blue cells. Transformation efficiency of the cells with pUC19 is 0.1-1x10^7 cfu/µg of supercoiled DNA. Cloning efficiency of the Control PCR Product into the pJET1.2/blunt exceeded 0.1-1x10^5 cfu/µg of vector DNA. More than 99% of the recombinant plasmids contained the appropriate insert. Each lot of the pJET1.2 Forward Sequencing Primer and pJET1.2 Reverse Sequencing Primer was functionally tested in DNA sequencing and in colony PCR.

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.

Trademarks

PRODUCT USE LIMITATION.

This product is developed, designed and sold exclusively for research purposes and *in vitro* use only. The product was not tested for use in diagnostics or for drug development, nor is it suitable for administration to humans or animals.

Please refer to www.fermentas.com for Material Safety Data Sheet of the product.

RELATED PRODUCTS

Product	Amount	Catalog #
Pfu DNA Polymerase, native*	100 u 500 u	EP0571 EP0572
Pfu DNA Polymerase, recombinant*	100 u 500 u	EP0501 EP0502
DreamTaq™ DNA Polymerase , 5 u/µl	200 u 500 u 5 x 500 u 20 x 500 u	EP0701 EP0702 EP0703 EP0704
Taq DNA Polymerase, recombinant, 5 u/µl	100 u 500 u	EP0401 EP0402
2X PCR Master Mix	2 x 1.25 ml	K0171
TrueStart™ *Taq* DNA Polymerase, 5 u/µl	100 u 500 u	EP0611 EP0612
PyroStart™ Fast PCR Master Mix (2X)	2 x 1.25 ml	K0211
High Fidelity PCR Enzyme Mix*	100 u 500 u	K0191 K0192
Long PCR Enzyme Mix*	100 u 500 u	K0181 K0182
dNTP Mix, 2 mM each	1 ml 5 ml	R0241 R0242
dNTP Mix, 10 mM each	0.2 ml 1 ml 5 x 1 ml	R0191 R0192 R0193
dNTP Set, 100 mM	4 x 0.25 ml 4 x 1 ml 4 x 5 ml	R0181 R0182 R0186
TransformAid™ Bacterial Transformation Kit	20 reaction 40 reactions	K2710 K2711
GeneJET™ Plasmid Miniprep Kit	50 preps 250 preps	K0502 K0503
DNA Extraction Kit	100 preps	K0513
TranscriptAid™ T7 High Yield Transcription Kit	50 reactions	K0441
T7 Transcription Kit	10 reactions 30 reactions	K0411 K0412
T7 RNA Polymerase	5000 u 5 x 5000 u 25000 u, HC	EP0111 EP0112 EP0113

*Not available in the USA.