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PRODUCT INFORMATION

Thermo Scientific InsTAclone PCR Cloning Kit

#K1213, #K1214

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www.thermofisher.com

For Research Use Only. Not for use in diagnostic procedures.

#_
Lot _

CERTIFICATE OF ANALYSIS

All components of the kit were function tested in control experiment as described in the manual. A 2.5 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 pTZ57R/T was >10^5 cfu/µg DNA.
>90% of the recombinant (white) colonies contained plasmids with the appropriate size insert.
Thermo Scientific™ TransformAid™ Bacterial Transformation Kit (T-Solutions (A) and (B) and C-Medium) was tested for transformation efficiency of *E. coli* strains XL1-Blue and JM107. Competent cells prepared with this kit were transformed with supercoiled pUC19 DNA with the efficiency of >10^6 cfu/µg DNA.

Quality authorized by:  Jurgita Zilinskiene

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COMPONENTS OF THE KIT

Component	#K1213 10 reactions	#K1214 30 reactions
Vector pTZ57R/T, 55 ng/µL	30 µL	90 µL
5X Ligation Buffer	100 µL	300 µL
T4 DNA Ligase, 5 u*/µL	15 µL	30 µL
Control PCR Fragment, 42 ng/µL 953 bp long	8 µL	20 µL
Control DNA 1, 0.1 µg/µL circular supercoiled plasmid vector pTZ57R DNA without insert, 2886 bp	12 µL	30 µL
Control DNA 2, 0.1 µg/µL circular supercoiled plasmid vector pTZ57R DNA with inserted control PCR fragment, 3839 bp	12 µL	30 µL
Water, nuclease-free	1.25 mL	1.25 mL
Components of TransformAid Bacterial Transformation Kit**		
C-Medium	35 mL	2 x 35 mL
T-Solution (A)	2 x 1.25 mL	4 x 1.25 mL
T-Solution (B)	2 x 1.25 mL	4 x 1.25 mL

* Weiss unit.
** Bacterial cells are not included.

STORAGE

All components of the kit should be stored at -20 °C. The C-Medium can be stored at 4 °C for up to two months or at -20 °C for longer periods.

DESCRIPTION

The Thermo Scientific™ InsTAclone™ PCR Cloning Kit is a TA system for direct one-step cloning of PCR products with 3'-dA overhangs (1). The high quality TA cloning vector pTZ57R/T is ready to use for efficient ligation with PCR products providing high cloning yields and low background. To increase the speed, convenience and efficiency of cloning, the InsTAclone PCR Cloning Kit has been combined with the TransformAid Bacterial Transformation Kit – a set of solutions for preparation of chemically competent *E. coli* cells. According to our protocol, ligation and preparation of competent cells is performed in parallel. Therefore, it only takes approximately one hour from the completion of PCR to plating of transformed cells. Our transformation protocol is often faster than transformation of commercially available competent cells.
The DNA insert can be readily excised from the versatile polylinker of pTZ57R/T, sequenced using standard M13/pUC primers or *in vitro* transcribed with T7 RNA polymerase.

CLONING PRINCIPLE

The InsTAclone PCR 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.

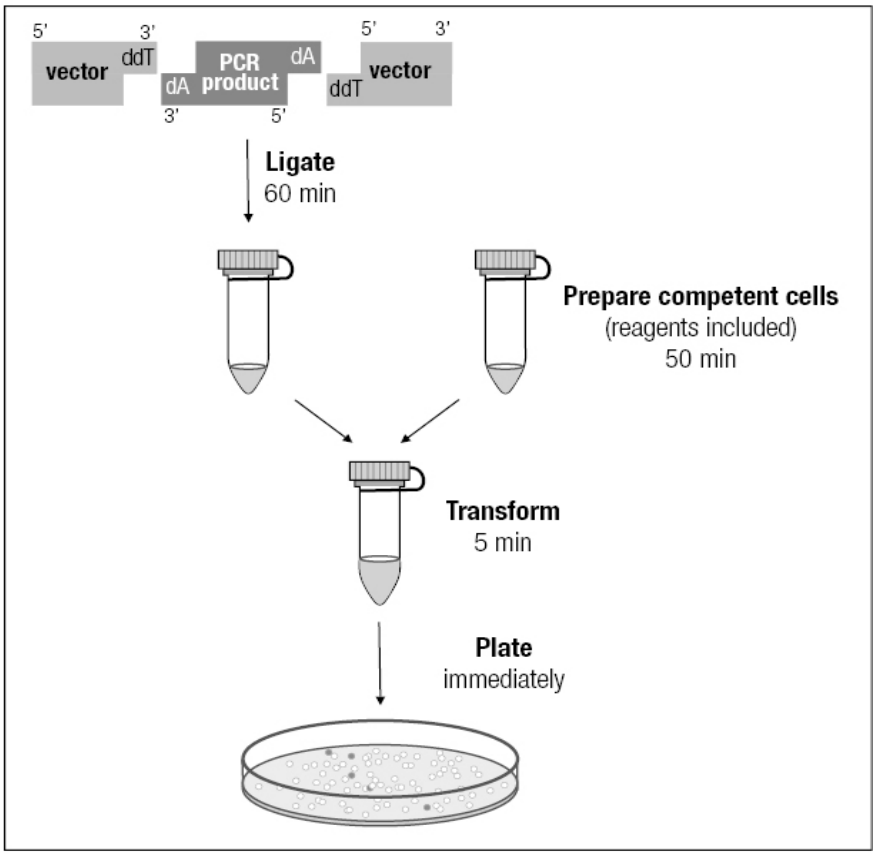

Fig. 1. PCR product cloning with InsTAclone PCR Cloning Kit.

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 InsTAclone 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 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 (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. (2,3).
- The optimal insert/vector ratio is 3:1. Refer to Table 1 to calculate the amount of PCR product, required for efficient ligation with 0.165 µg (3 µL, 0.172 pmol ends) of the pTZ57R/T vector or use dedicated software (www.thermofisher.com/reviewer) for calculations.

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

Length of PCR product (bp)	Optimal PCR product quantity for ligation reaction, (0.52 pmol ends)
100	17 ng
300	51 ng
500	86 ng
1000	172 ng
2000	343 ng
3000	515 ng
4000	686 ng
5000	858 ng

- Components of TransformAid Bacterial Transformation Kit allow for fast and efficient preparation of chemically competent cells using *E. coli* strain of choice. To enable blue/white screening, choose only strains having lacZ∆M15 mutation, for example, XL1-Blue, ER1727, JM109 or other.

CONSIDERATIONS FOR CLONING LONG PCR PRODUCTS

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.

- Optimize PCR conditions to increase specificity and yield of full length PCR product.
- Gel-purify PCR products (e.g., with GeneJET Gel Extraction Kit, #K0691) to minimize presence of primer dimmers or non-specific short PCR products in the ligation reaction, even if these are not visible on the gel.
- Protect long PCR products from mechanical sharing and damage by nucleases:
  – store the PCR product at -20 °C if it is not used immediately;
  – use clean labware, razor blade and electrophoresis tank. Prepare fresh electrophoresis running buffer for gel purification procedure.
- Avoid DNA damage by UV light (*see* IMPORTANT NOTES on page 4).
- Before ligation, verify the quantity and quality of the purified PCR product on a gel. The optimal insert/vector ratio is 3:1. Refer to Table 1 (page 4) to calculate the amount of PCR product (0.52 pmol ends), required for efficient ligation with 0.165 µg (3 µL, 0.172 pmol ends) of the pTZ57R/T vector or use dedicated software (www.thermofisher.com/reviewer) for calculations.
- 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 pTZ57R/T may not be suitable for cloning these PCR products.

CLONING PROTOCOL

Please read all IMPORTANT NOTES on page 4 before starting.

Ligation

1. Set up the ligation reaction:

Component	Volume
Vector pTZ57R/T, (0.17 pmol ends)	3 µL
5X Ligation Buffer	6 µL
PCR product (0.52 pmol ends)	variable*
Water, nuclease-free	to 29 µL
T4 DNA Ligase	1 µL
Total volume	**30 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 1 hour. 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 – TransformAid 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 2.5 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.

Transformation

The TransformAid Bacterial Transformation Kit is provided for fast and efficient preparation of chemically competent cells and fast transformation. *E. coli* cells are not included in this kit. All common *E. coli* laboratory strains can be used. To enable blue/white screening, choose only strains having lacZ∆M15 mutation, for example, XL1-Blue, ER1727, JM109 or other. Typical transformation efficiencies are more than 10^7 transformants per µg of supercoiled plasmid DNA. The TransformAid Bacterial Transformation Kit provides you flexibility in using either overnight liquid bacterial culture or bacterial colonies from agar plates for preparation of competent cells.

- Use overnight culture to prepare competent cells in only 50 min. Following this, the transformation takes only 5 minutes and the cells can be plated immediately. There is no need for heat shock or an incubation of transformed cells in SOC medium.
- Use bacterial colonies, if overnight culture is not available. The procedure takes 2.5 hours but nevertheless it allows researchers to transform *E. coli* strain of choice the same day.

Note

- All procedures are performed on ice. All short centrifugations can be carried out at room temperature (RT) in a regular benchtop centrifuge. Do not keep cells in the centrifuge at RT for more than 5 min as this will significantly decrease the transformation efficiency.
- Competent cells prepared with TransformAid Bacterial Transformation Kit are suitable for direct use only. Freezing down and storage at -70 °C is not recommended.

Transformation Protocol from Overnight Bacterial Culture (for 2 transformations)

- The day before the transformation seed overnight culture by inoculating 2 mL of C-medium with a single bacterial colony. Make sure to use freshly streaked bacterial colonies (not older than 10 days*). Incubate the culture overnight at 37 °C in a shaker.
- 2 mL of overnight culture is sufficient for 26 transformations. The culture can be kept at 4 °C for one week and used for preparation of competent cells.
- The day of transformation pre-warm culture tubes containing the required amount of C-medium (1.5 mL for each 2 transformations) at 37 °C for at least 20 min. Pre-warm LB agar plates, supplemented with ampicillin, X-Gal and IPTG (*see* recipes on p. 15) in a 37 °C incubator for at least 20 min before plating.
- Prepare T-solution: thaw T-solution (A) and T-solution (B), mix contents thoroughly. Combine 250 µL of T-solution (A) and 250 µL of T-solution (B) in a separate tube and keep on ice.

Step	Procedure
1	Add 150 µL of the overnight bacterial culture to 1.5 mL of pre-warmed C-medium. Incubate 20 min at 37 °C in a shaker.
2	Pellet bacterial cells by 1 min centrifugation, discard the supernatant.
3	Resuspend cells in 300 µL of T-solution. Incubate on ice for 5 min.
4	Centrifuge for 1 min in a microcentrifuge, discard the supernatant.
5	Resuspend pelleted cells in 120 µL of T-solution. Incubate 5 min on ice.
6	Add 2.5 µL of ligation mixture (containing 14 ng vector DNA) or 1 µL of supercoiled Control DNA (10-100 pg) into new microcentrifuge tubes. Chill on ice for 2 min.
7	Add 50 µL of the prepared cells to each tube containing DNA, mix and incubate on ice for 5 min.
8	Plate immediately on pre-warmed LB-ampicillin X-Gal/IPTG agar plates. Incubate overnight at 37 °C.

**E. coli* DH5α™ should only be <24 hours fresh.

Transformation Protocol from Bacterial Colonies (for 2 transformations)

- Seed an LB plate with a single bacterial colony using the streak method and incubate the plate overnight at 37 °C. Use freshly streaked bacterial colonies (not older than 10 days*), for preparation of competent cells.
- Before the transformation pre-warm culture tubes containing the required amount of C-medium (1.5 mL for each 2 transformations) at 37 °C for at least 20 min. Pre-warm LB agar plates, supplemented with ampicillin, X-Gal and IPTG (*see* recipes on p. 15) in a 37 °C incubator for at least 20 min before plating.
- Prepare T-solution: thaw T-solution (A) and T-solution (B), mix contents thoroughly. Combine 250 µL of T-solution (A) and 250 µL of T-solution (B) in a separate tube and keep on ice.

Step	Procedure
1	Transfer a portion of freshly streaked bacterial culture (4 x 4 mm size) to 1.5 mL of pre-warmed C-medium using an inoculating loop. Suspend the cells by gently mixing and incubate the tubes at 37 °C for 2 hours in a shaker.
2	Pellet bacterial cells by 1 min centrifugation, discard the supernatant.
3	Resuspend cells in 300 µL of T-solution. Incubate on ice for 5 min.
4	Centrifuge for 1 min in a microcentrifuge, discard the supernatant.
5	Resuspend pelleted cells in 120 µL of T-solution. Incubate 5 min on ice.
6	Add 2.5 µL of ligation mixture (containing 14 ng vector DNA) or 1 µL of supercoiled Control DNA (10-100 pg) into new microcentrifuge tubes. Chill on ice for 2 min.
7	Add 50 µL of the prepared cells to each tube containing DNA, mix and incubate on ice for 5 min.
8	Plate immediately on pre-warmed LB-ampicillin X-Gal/IPTG agar plates. Incubate overnight at 37 °C.

**E. coli* DH5α should only be <24 hours fresh.

Scaling up

To prepare larger volumes of competent cells for transformation the procedure can be scaled up by increasing volumes of all components accordingly. In steps 2 and 4 the cells are centrifuged in large centrifuge tubes at 5000-10000 x g for 5 min at 4 °C.

ANALYSIS OF RECOMBINANT CLONES

Analyze 4-6 white 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	–
M13/pUC sequencing primer, 10 µM	0.6 µL	0.6 µL
M13/pUC reverse sequencing primer, 10 µM	0.6 µL	0.6 µL
Taq DNA polymerase 5 U/µL, #EP0401 *or* Thermo Scientific™ DreamTaq™ Green DNA Polymerase, #EP0711	0.1 µL	–
PCR Master Mix (2X), #K0171 *or* DreamTaq Green PCR Master Mix (2X), #K1081	–	10 µL
Water, nuclease-free	to 20 µL	to 20 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 white colony and resuspend in 20 µL of the PCR master mix. Make a short strike over culture plate to save the clone for repropagation.
4. Perform PCR: 94 °C, 2 min; 94 °C, 30 s, 45 °C*, 30 s, 72 °C 1 min/kb; 30 cycles.
   * Depends on primer melting temperature (Tm-5).
5. Analyze on a gel for the presence of the PCR product of the expected length.

Note. Due to considerable amount of recircularized 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.

Restriction analysis

Isolate plasmid DNA from an overnight bacterial culture using a convenient plasmid miniprep method. To speed up the process and to assure the quality of purified plasmid DNA, use the GeneJET Plasmid Miniprep Kit (#K0502). To digest DNA from recombinant clones in just 5 minutes, use Thermo Scientific FastDigest restriction enzymes.

Sequencing

Isolate plasmid DNA from an overnight bacterial culture using a reliable plasmid miniprep method. To assure the sequencing quality of purified plasmid DNA, use the GeneJET Plasmid Miniprep Kit (#K0502). Sequence the insert using standard M13/pUC sequencing primers (#SO100, #SO113, #SO101, #SO114) or T7 promoter sequencing primer (#SO118).

CONTROL EXPERIMENT

The Control PCR Fragment is a 953 bp purified amplicon generated with *Taq* DNA polymerase, which adds extra nucleotides to the 3'-ends of PCR products.

1. Set up the ligation reaction:

Component	Volume
pTZ57R/T Cloning vector, (0.17 pmol ends)	3 µL
5X Ligation Buffer	6 µL
Control PCR Fragment (0.52 pmol ends)	4 µL
Water, nuclease-free	16 µL
T4 DNA Ligase	1 µL
Total volume	**30 µL**

Vortex briefly and centrifuge for 3-5 s.

2. Incubate the ligation mixture at room temperature (22 °C) for 1 hour. Use 2.5 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, prepared with TransformAid Bacterial Transformation Kit, with 2.5 µL of the ligation mixture according to protocol on p. 6.

   In parallel check the transformation efficiency of competent cells by transforming 50 µL of cells with 0.1 ng of a supercoiled circular plasmid, e.g., Control DNA 1 or 2 (supplied with the kit).

4. Plate cells on LB-ampicillin X-Gal/IPTG agar plates (*see* p.15 for recipes).

   Note. Transformation with Control DNA 1 (vector without insert) will yield all blue colonies, and control transformation with Control DNA 2 (vector with inserted control PCR fragment) will yield all white colonies.

Results of control cloning experiment

Transformation efficiency of *E. coli* competent cells should exceed 10^7 cfu/µg. Control cloning experiment should yield 200-1000 colonies, depending on the *E. coli* strain used. Approximately 90% colonies should be white.
Analyze 10 white colonies for the presence of 953 bp insert using your preferred analysis method. Control DNA 2 (the cloning vector with inserted control PCR fragment) can be used as a positive control, Control DNA 1 (vector without insert) can be used as negative control.
At least 9 of 10 analyzed colonies should contain the plasmid with the insert.

MAP AND FEATURES OF pTZ57R/T CLONING VECTOR

The pTZ57R/T cloning vector is linearized and ddT tailed for direct use in cloning of PCR products, generated with *Taq, Tth, Tfl* or other DNA polymerases or polymerase mixtures, which add extra adenines to the ends of PCR products.
The map and the MCS region of the vector are presented in Fig.2 and Fig. 3.
pTZ57R/T vector sequence is available for downloading at www.thermofisher.com.

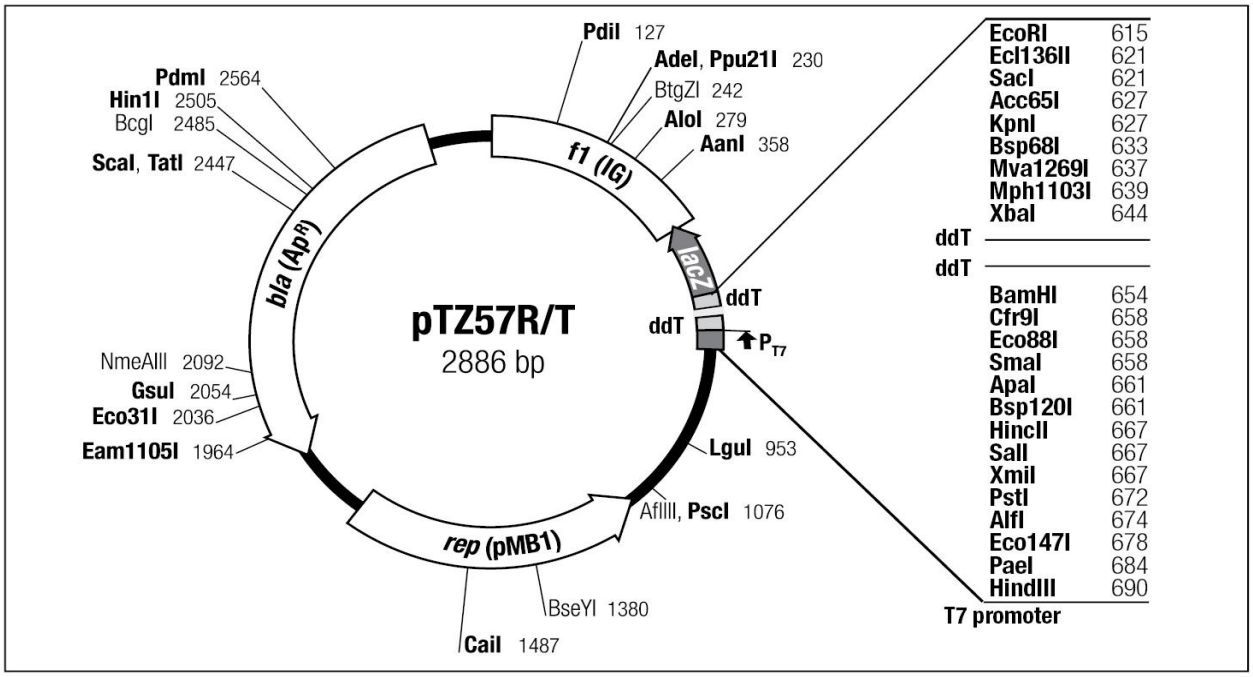

Fig. 2. Map of the pTZ57R/T cloning vector. Unique restriction sites are indicated.

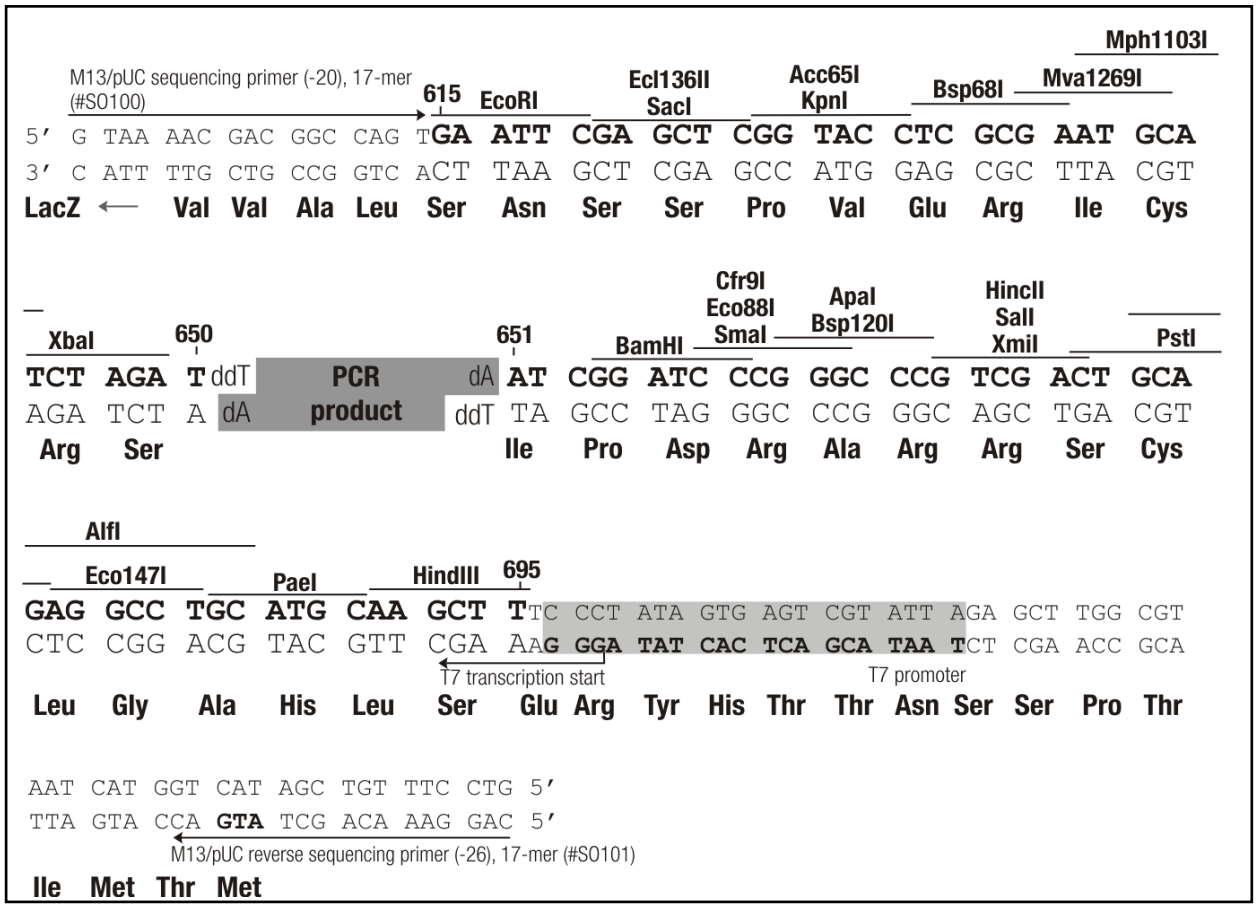

Fig. 3. DNA sequence of MCS region.

Genetic elements of pTZ57R/T cloning vector

Element	Function	Position (bp)
rep (pMB1)	A replicon (rep) from the pMBI plasmid is responsible for the replication.	1122-1736
Replication start	Initiation of the replication.	1136 (±1)
bla (Ap^R)	β-lactamase gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells.	1896-2756
LacZ α-peptide	Blue/white screening of recombinant clones	449-739
Multiple cloning site (MCS)	Mapping, screening and excision of the cloned insert	615-695
Cloning site	3'-ddT tailed DNA ends for ligation with insert	650-651
Phage f1 origin	Synthesis of a single-stranded DNA	2-457
T7 promoter	*In vitro* transcription of insert DNA with T7 RNA polymerase	697-716
M13/pUC forward sequencing primer (-20)	Sequencing of insert, colony PCR	599-614
M13/pUC reverse sequencing primer (-26)	Sequencing of insert, colony PCR	735-751
T7 promoter sequencing primer	Sequencing of insert, colony PCR	697-716

Restriction enzymes, that do not cut pTZ57R/T:

AarI, AatII, AbsI, AjiI, AjuI, BaeI, BbvCI, BclI, BcuI, BglII, BoxI, BpiI, BplI, Bpu10I, Bpu1102I, BseJI, BseRI, BsgI, BshTI, Bsp119I, Bsp1407I, BspOI, BspTI, Bst1107I, BstAPI, BstXI, Bsu15I, BtgI, BveI, Cfr42I, CpoI, CspCI, Eco32I, Eco47III, Eco52I, Eco72I, Eco81I, Eco91I, Eco105I, Eco130I, EcoO109I, EheI, Esp3I, FalI, FaqI, FseI, FspAI, Kpn2I, KspAI, MauBI, MLsI, MLuI, MreI, MssI, MunI, NcoI, NdeI, NheI, NotI, OliI, PacI, PasI, PauI, Pfl23II, PfoI, Psp5II, PspXI, PsrI, PsyI, SanDI, SdaI, SexAI, SfaI, SfiI, SgfI, SgrAI, SgrDI, SgsI, SmiI, SrfI, TstI, Van91I, XagI, XcmI, XhoI, XmaJI.

Restriction enzymes, that cut pTZ57R/T once:

AanI	358	Bsp120I*	661	Hin1I	2505	Ppu21I	230
Acc65I*	627	BtgZI	242	HincII*	667	PscI	1076
AdeI	230	CaiI	1487	HindIII*	690	PstI*	672
AflIII	1076	Cfr9I*	658	KpnI*	627	SacI*	621
AlfI*	674	Eam1105I	1964	LguI	953	SalI*	667
AloI	279	Ecl136II*	621	Mph1103I*	639	ScaI	2447
ApaI*	661	Eco31I	2036	Mva1269I*	637	SmaI*	658
BamHI*	654	Eco88I*	658	NmeAIII	2092	TatI	2447
BcgI	2485	Eco147I*	678	PaeI*	684	XbaI*	644
BseYI	1380	EcoRI*	615	PdiI	127	XmiI*	667
Bsp68I*	633	GsuI	2054	PdmI	2564	* – MCS	

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 DNA 1 (supercoiled pTZ57R 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. Alternatively, for cloning of blunt PCR products use Thermo Scientific CloneJET PCR Cloning Kit (#K1231). **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. (2,3). **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 pTZ57R/T vector or use dedicated software (www.thermofisher.com/reviewer) 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 (*see* p.14 for recipes).
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 the PCR reaction, gel-purify the target PCR product. Otherwise, optimize the PCR conditions to increase specificity.

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 (2, 3). **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) (#R0404) in 10 mL N,N-dimethylformamide. Store at -20 °C in the dark. Alternatively, use X-Gal Solution, ready-to-use (#R0941). Use 40 µL per plate.

IPTG stock solution (100 mM)

Dissolve 1.2 g IPTG (isopropyl- β -D-thiogalactopyranoside) (#R0392) in 50 mL deionized water. Filter-sterilize, aliquote and store at 4 °C. Alternatively, use IPTG Solution, ready-to-use (#R1171). Use 40 µL per plate.

LB-ampicillin X-Gal/IPTG 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 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 (#R0941) and 40 µL of IPTG 100 mM or IPTG Solution, ready-to-use (#R1171), spread evenly with a sterile spatula.

References

1. Clark, J.M., Novel non-templated nucleotide addition reactions catalyzed by prokaryotic and eukaryotic DNA polymerases, Nucl. Acids Res., 16 (20), 9677-9686, 1988.
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