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PRODUCT INFORMATION

Thermo Scientific aLICator Ligation Independent Cloning and Expression System

aLICator LIC Cloning and Expression Kit 1 (untagged), **#K1241**

aLICator LIC Cloning and Expression Kit 2 (N-terminal His-tag/EK), **#K1251**

aLICator LIC Cloning and Expression Kit 3 (C-terminal His-tag), **#K1261**

aLICator LIC Cloning and Expression Kit 4 (N-terminal His-tag/WQ), **#K1281**

aLICator LIC Cloning and Expression Set 1 (All-in-One/EK), **#K1271**

aLICator LIC Cloning and Expression Set 2 (All-in-One/WQ), **#K1291**

www.thermoscientific.com/onebio

#K1281
Lot
Expiry Date

CERTIFICATE OF ANALYSIS

Kit components were functionally tested in control experiments as outlined in the manual.
A 2.5 µL aliquot of the LIC 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 LIC vector was >4×10^4 cfu/µg DNA and >95% of the recombinant plasmids contained the appropriate insert.

Quality authorized by: Jurgita Zilinskiene

Rev.5 .

CONTENTS page

KIT COMPONENTS

Components	aLICator LIC Cloning and Expression Kits				aLICator LIC Cloning and Expression Sets	
	Kit 1 untagged	Kit 2 N-terminal His-tag/EK	Kit 3 C-terminal His-tag	Kit 4 N-terminal His-tag/WQ	Set 1 All-in-One/ EK	Set 2 All-in-One/ WQ
	#K1241 20 rxns	**#K1251 20 rxns**	**#K1261 20 rxns**	**#K1281 20 rxns**	**#K1271 3×10 rxns**	**#K1291 3×10 rxns**
pLATE11 vector,	25 µL	___	___	___	15 µL	15 µL
pLATE51 vector	___	25 µL	___	___	15 µL	___
pLATE52 vector	___	___	___	25 µL	___	15 µL
pLATE31 vector	___	___	25 µL	___	15 µL	15 µL
5 x LIC Buffer	100 µL	100 µL	100 µL	100 µL	100 µL	100 µL
T4 DNA polymerase	24 µL	24 µL	24 µL	24 µL	36 µL	36 µL
Water, nuclease-free	1.25 mL	1.25 mL	1.25 mL	1.25 mL	1.25 mL	1.25 mL
0.5 M EDTA, pH 8.0	1 mL	1 mL	1 mL	1 mL	1 mL	1 mL
LIC-forward sequencing primer	100 µL	100 µL	100 µL	100 µL	150 µL	150 µL
LIC-reverse sequencing primer	100 µL	100 µL	100 µL	100 µL	150 µL	150 µL
pLATE31-Cm control plasmid DNA	10 µL	10 µL	10 µL	10 µL	10 µL	10 µL
Control PCR fragment for pLATE11 vector	10 µL	___	___	___	10 µL	10 µL
Control PCR fragment for pLATE51 vector	___	10 µL	___	___	10 µL	___
Control PCR fragment for pLATE52 vector	___	___	___	10 µL	___	10 µL
Control PCR fragment for pLATE31 vector	___	___	10 µL	___	10 µL	10 µL

STORAGE

All components of the *Thermo Scientific aLICator LIC Cloning and Expression Kits* should be stored at -20°C.

OVERVIEW

The aLICator™ LIC Cloning and Expression System is designed for fast and efficient ligation independent cloning and tight regulation of gene expression in *E. coli*. The pLATE bacterial expression vectors are designed for high levels of target protein expression in concert with minimal basal (uninduced) expression, which permits expression of proteins that are toxic to *E. coli* cells. To streamLine and facilitate the process of insert cloning into the expression vector, the aLICator system uses directional LIC cloning technology, a rapid procedure that provides high cloning efficiencies.

Tightly regulated expression and fast, efficient directional cloning makes the aLICator LIC Cloning and Expression System the best choice for routine and toxic gene cloning and expression in *E. coli*.

The system consists of six kits based on the pLATE series of bacterial expression vectors:

1. aLICator LIC Cloning and Expression Kit 1 - pLATE11 vector, untagged protein expression.
2. aLICator LIC Cloning and Expression Kit 2 (N-terminal His-tag/EK) – pLATE51 vector, N-terminal His-tag protein expression, enterokinase cleavage.
3. aLICator LIC Cloning and Expression Kit 3 (C-terminal His-tag) – pLATE31 vector, C-terminal His-tag protein expression.
4. aLICator LIC Cloning and Expression Kit 4 (N-terminal His-tag/WQ) – pLATE52 vector, N-terminal His-tag protein expression, WELQut cleavage.
5. aLICator LIC Cloning and Expression Set 1 (All-in-One/EK) – pLATE11, pLATE51 and pLATE31 vectors, choice of untagged, N- or C-terminal His-tag protein expression, enterokinase cleavage.
6. aLICator LIC Cloning and Expression Set 2 (All-in-One/WQ) – pLATE11, pLATE52 and pLATE31 vectors, choice of untagged, N- or C-terminal His-tag protein expression, WELQut cleavage.

For proteins with a known preference for either the N- or C-terminal 6xHis-tag position, using the appropriate N- or C-terminal kit is recommended. Cleavage sites of two different proteases are located immediately N-terminal to the target protein. Following protein affinity purification, amino-terminal tags can be removed either via enterokinase (EK), DDDDK^ or a novel protease WELQut (WQ), WELQ^. This new protease has several procedural advantages - it is suitable for on-column proteolysis and can easily be removed from the cleavage reaction during affinity purification using its built-in His-tag.

Both proteases, EK and WELQut, cleave outside the recognition sequence without leaving additional amino acids bound to the target protein.

When the protein structure and features are not well-known, it is recommended to use one of the Set Kits (see Table 1). The most compatible vector for downstream applications can be determined by cloning into all three vectors supplied with the Set Kit – for untagged, C-tagged, N-tagged/EK or N-tagged/WQ protein expression.

Table 1. Overview of the aLICator LIC Cloning and Expression System

aLICator LIC Cloning and Expression Kit	Vector name	6xHis-tag	Protease cleavage site	Main application
Kit 1 (untagged) #K1241	pLATE11	No	No	Bacterial expression of untagged proteins
Kit 2 (N-terminal His-tag/EK) #K1251	pLATE51	N-terminal	Enterokinase (EK), DDDDK^	Bacterial expression of proteins with N-terminal 6xHis-tag and enterokinase cleavage site
Kit 3 (C-terminal His-tag) #K1261	pLATE31	C-terminal	No	Bacterial expression of proteins with C-terminal 6xHis-tag
Kit 4 (N-terminal His-tag/WQ) #K1281	pLATE52	N-terminal	WELQut (WQ), WELQ^	Bacterial expression of proteins with N-terminal 6xHis-tag and WELQut Protease cleavage site
Set 1 (All-in-One/EK) #K1271	pLATE11 pLATE51 pLATE31	Set of three vectors	Enterokinase in pLATE51	Cloning of the target gene into three vectors with different 6xHis-tag positions
Set 2 (All-in-One/WQ) #K1291	pLATE11 pLATE52 pLATE31	Set of three vectors	WELQut in pLATE52	Cloning of the target gene into three vectors with different 6xHis-tag positions

Main features of pLATE bacterial expression vectors include:

- High yields of expressed proteins driven by the bacteriophage T7 promoter
- High efficiency cloning and tight control of gene expression:
  - *lac* operators placed downstream and upstream of the T7 promoter
  - rrnBT1-T2 terminator and Tet promoter flanking the T7 promoter
  - Controlled expression of the cloned gene

pLATE expression vectors use elements from bacteriophage T7 to control expression of heterologous genes in *E. coli*. The expression of the gene of interest is driven by a strong bacteriophage T7 promoter that is specifically recognized by T7 RNA polymerase. To express the gene of interest, *E.coli* strains such as BL21 (DE3), HMS 174 (DE3) must be used, in which expression of the T7 RNA polymerase gene is under the control of an inducible promoter such as *lacUV5.* After IPTG induction, T7 RNA polymerase is expressed within the cell, and begins transcription of genes under the T7 promoter.

Studies have shown that expression of T7 RNA polymerase from the *lacUV5* promoter in λDE3 lysogens is leaky, even in the absence of an inducer (1). If the gene of interest is toxic to the *E. coli* host, basal expression of the gene may lead to plasmid instability and/or cell death. To overcome this problem, pLATE vectors contain additional elements that reduce basal T7 RNA polymerase expression 2–2.5 fold. These elements include two *lac* operators that flank the T7 promoter and serve as binding sites for the lac repressor (encoded by the *lacI* gene).

A rrnBT1-T2 terminator is also placed upstream of the cloning site to prevent basal gene expression from vector derived promoter-like elements. In addition, a constitutively-induced

weak Tet promoter (P_{tet}) operates in the opposite direction to the T7 promoter, further reducing basal expression.
Target genes are directionally cloned into the pre-linearized pLATE vectors. Following (or during) protein affinity purification, amino-terminal tags can be removed via either enterokinase (DDDDK^) or a novel WELQut protease (WELQ^) cleavage sites located immediately N-terminal to the target protein.
Inserts can be conveniently subcloned using rare-cutting restriction enzymes via recognition sequences present on both sides of the LIC cloning site.

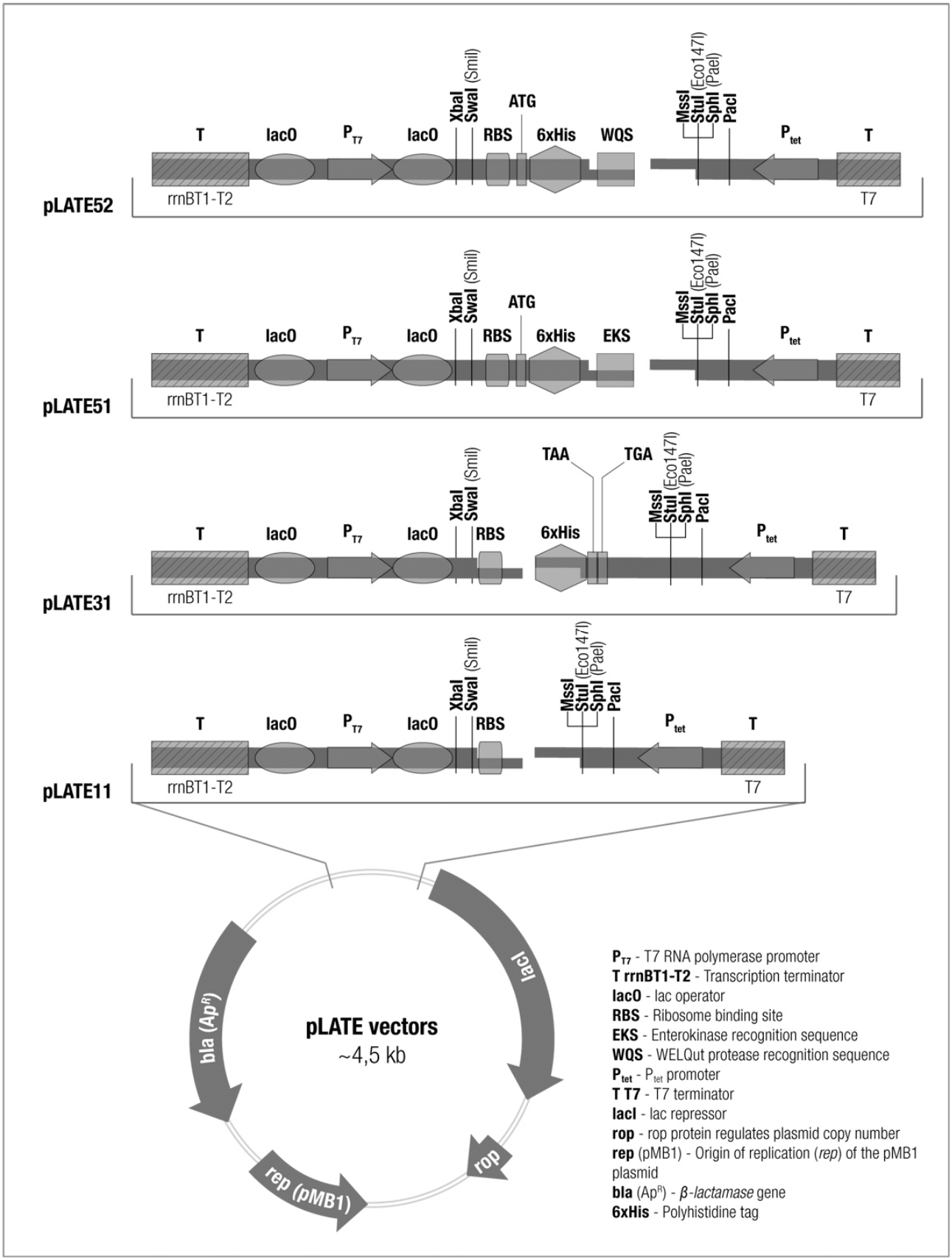

Figure 1. pLATE vector elements.

Genetic elements of pLATE cloning vectors

Element	Function
T rrnBT1-T2	Transcription terminator rrnBT1-T2 prevents basal gene expression from vector derived promoter-like elements
P_{T7} lacO	The T7 RNA polymerase promoter, which drives transcription of the cloned gene, and two flanking lac operator sequences ensure tight control of gene expression
RBS	Ribosome binding site for effective gene translation
EKS	Enterokinase recognition sequence
WQS	WellQut protease recognition sequence
ATG	Start codon
TAA, TGA	Stop codons
P_{tet}	The P_{tet} promoter reduces basal expression from the T7 promoter
T T7	The T7 terminator terminates transcription from the T7 promoter
lacI	The lac repressor ensures tight control of basal expression from the T7 promoter
rop	The rop protein regulates plasmid copy number
rep (pMB1)	Origin of replication (*rep*) of the pMB1 plasmid
bla (Ap^R)	*β-lactamase* gene conferring resistance to ampicillin. Used for selection and maintenance of recombinant *E.coli* cells
6xHis	N- or C-terminal polyhistidine tag

Primer sequences

LIC Forward Sequencing primer, 20-mer	5'- TAATACGACTCACTATAGGG -3'
LIC Reverse Sequencing primer, 24-mer	5'- GAGCGGATAACAATTTCACACAGG-3'

Restriction enzymes sites in pLATE vectors

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

LIC CLONING PRINCIPLE

The aLICator LIC cloning system uses directional LIC cloning technology to streamLine and facilitate cloning into an expression vector. LIC ensures high cloning efficiencies of more than 95% and eliminates the need for ligation and restriction enzyme digestion steps.
The LIC method uses T4 DNA polymerase to create specific 14–21 nucleotide single-stranded overhangs on the pLATE vectors and DNA inserts (2). T4 DNA polymerase has two enzymatic activities: 5'–3' polymerase activity and 3'–5' exonuclease activity. The exonuclease activity removes nucleotides from the 3' ends of the DNA while the polymerase activity restores the chain using dNTPs and the complementary DNA strand as a template. In the LIC protocol, only dGTP is included in the reaction, causing the 3'-5' exonuclease and
5'-3' polymerase activities to equilibrate at the first occurrence of cytosine in the complementary strand (Fig. 2). After annealing, the LIC vector and insert are transformed into competent *E. coli* cells without the use of T4 DNA ligase. Covalent bond formation at the vector-insert junctions occurs within the cell to yield circular plasmid.

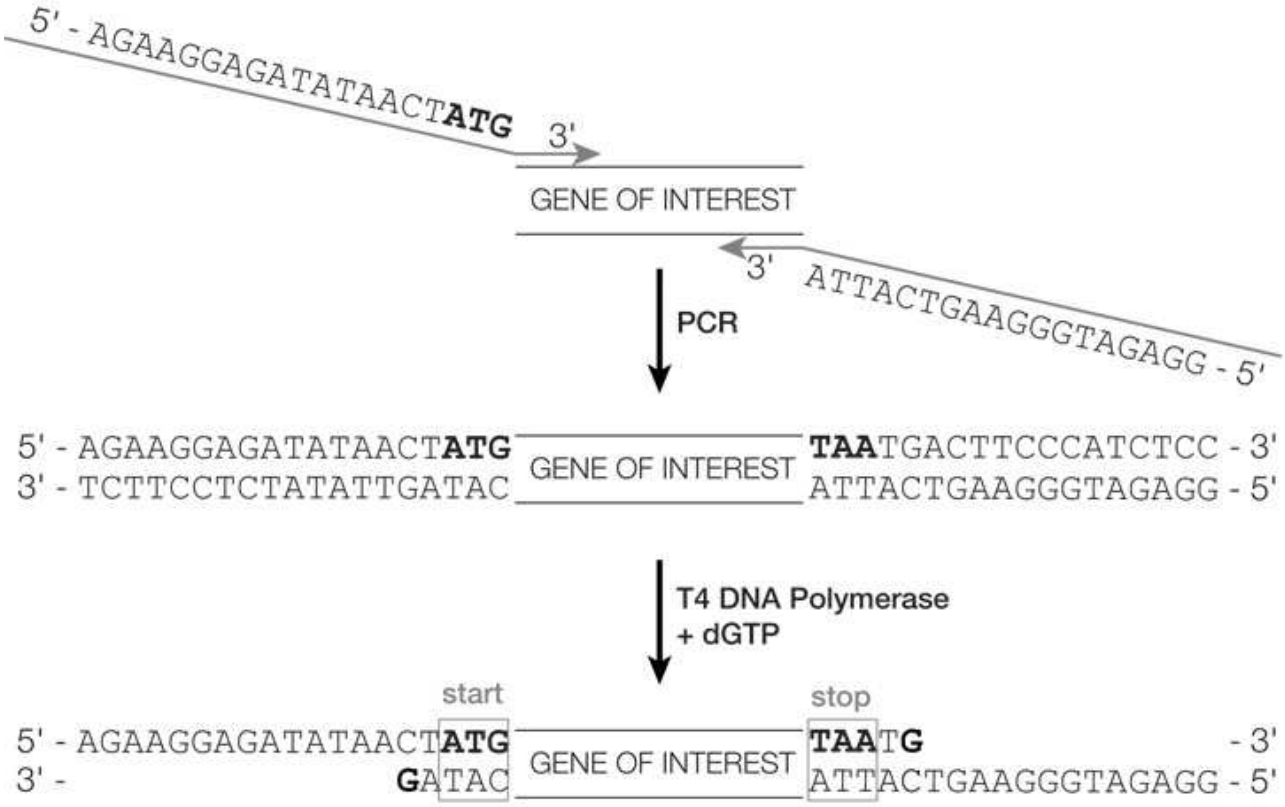

Figure 2. Generation of sticky ends on the gene of interest with T4 DNA polymerase and dGTP.

IMPORTANT NOTES

A. Designing PCR primers

Design PCR primers for the gene of interest according the recommendations outlined in Table 2. Ensure that the 5'-end of the primer includes vector-specific sequences and contains sufficient 3'-end overlap with the gene of interest. The melting temperature of the section of the primers complimentary to the gene of interest should be 60°C or more.
If Phusion® High Fidelity DNA Polymerase will be used for the PCR reaction, please visit www.thermoscientificbio.com/webtools/tmcalculator/ to obtain the correct annealing temperature to be used with this enzyme.

Table 2. **LIC vectors and primer design recommendations**

LIC Vector	6xHis-tag	Protease cleavage site	Primer
pLATE11	-	-	pLATE11 forward primer:* 5'-AGAAGGAGATATAACT**ATG**–*insert-specific sequence* 3' pLATE11 reverse primer:** 5'-GGAGATGGGAAGTCA**TTA**–*insert-specific sequence* 3'**,**
pLATE31	C- terminal	-	pLATE31 forward primer:* 5'- AGAAGGAGATATAACT**ATG**–*insert-specific sequence* 3' pLATE31 reverse primer: 5'-GTGGTGGTGATGGTGATGGCC –*insert-specific sequence* 3'
pLATE51	N- terminal	Enterokinase (DDDDK^)	pLATE51 forward primer: G D D D D K 5'–GGT GAT GAT GAT GAC AAG -*insert-specific sequence* 3' pLATE51 reverse primer:** 5' GGAGATGGGAAGTCA**TTA**–*insert-specific sequence* 3'
pLATE52	N- terminal	WELQut (WELQ^)	pLATE52 forward primer: G W E L Q 5'–GGT TGG GAA TTG CAA -*insert-specific sequence* 3' pLATE52 reverse primer:** 5' GGAGATGGGAAGTCA**TTA**–*insert-specific sequence* 3'

ATG are the first 3 nucleotides (start codon) of the insert-specific sequence on the forward primer, and if desired can be changed to alternative start codons GTG or TTG.
TTA are the last 3 nucleotides (stop codon) of the insert-specific sequence on the reverse primer.

* the same forward primer can be used as sense primer for insert cloning into pLATE11 and pLATE31 vectors.

** the same reverse primer can be used as antisense primer for insert cloning into pLATE11, pLATE51and pLATE52 vectors.

Note: *The "insert-specific sequence" referred to in the description for the forward primer indicates the sense DNA sequence immediately 3′ from the native start codon, but* ***not including*** *ATG itself.*
The "insert-specific sequence" referred to in the description for the reverse primer indicates the sequence complimentary to the 3′ end of the sense strand, but ***not including*** *the stop codon.*

B. PCR amplification of insert DNA

- Use a high fidelity DNA polymerase with proofreading activity, such as Phusion High-Fidelity DNA Polymerase (#F530S/L) to amplify insert DNA. Phusion High-Fidelity DNA Polymerase has the highest fidelity of any commercially available thermostabile polymerases and generates templates with accuracy and speed unattainable with a single enzyme. For the convenience of room-temperature reaction set up, Phusion Hot Start II High-Fidelity DNA Polymerase (#F549S/L) can be substituted for Phusion High-Fidelity DNA Polymerase.
- To further reduce the probability of amplification errors, use less than 25 PCR amplification cycles. Only 0.05–0.1 pmol of PCR product (32-65 ng of a 1,000 bp insert) is required per LIC reaction.
- For optimal PCR results, use HPLC-purified primers. Because the LIC system is ligation independent, 5′ phosphorylation of the primers is not necessary.
- Proofreading DNA polymerases do not incorporate dUTP, therefore do not use dNTP mixes that include dUTP. Use a high quality dNTP set (#R0181) or mix (#R0192) that does not contain trace amounts of dUTP.

C. Purification of PCR products

- It is essential to purify the PCR product to completely remove unincorporated dNTPs and primers, to inactivate the DNA polymerase and to remove contaminating DNA.
- Gel purification of the PCR product (e.g. with the Thermo Scientific GeneJET Gel Extraction Kit, #K0691) is strongly recommended for the best cloning results, especially if non-specific products are present in the PCR reaction or if the PCR template used was plasmid DNA with the same antibiotic resistance marker as the pLATE vector.
- If non-specific products or interfering template plasmids are not present in the PCR reaction, the agarose gel purification step is not necessary. In these cases, the PCR product can be purified by spin column clean-up (e.g. GeneJET PCR Purification Kit, #K0701).
- We recommend the following steps to help avoid damaging the PCR product:
  - Use PCR products immediately in the LIC protocol or store at -20°C.
  - Use clean labware, razor blades and electrophoresis tanks. Prepare fresh electrophoresis running buffer for the gel purification procedure.
- Avoid DNA damage by 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 or plastic plate during UV illumination. Alternatively, use dyes, such as crystal violet, to visualize DNA in ambient light (3, 4).

D. Calculating amounts of PCR product to use in the LIC reaction

- The quantity and quality of the purified PCR product has to be determined either by agarose gel electrophoresis or spectrophotometrically using the absorbance at 260 nm (assuming A_{260} =1 is 50 ng/µL).
  For spectrophotometric analysis of the PCR product, we recommend using the Thermo Scientific Nanodrop instrument.
  To calculate the DNA concentration in pmol/µl, use the following formula:
    - number of base pairs × 0.65 = ng/pmol

  Alternatively; use dedicated software such as;
  www.thermoscientificbio.com/webtools/reviewer/, to determine the pmol/µl value.
  For example, 65 ng of a 1000 bp DNA fragment is equivalent to 0.1 pmol.

Table 3. Recommended amount of PCR product for the LIC reaction.

Length of PCR product (bp)	Optimal PCR product quantity for LIC reaction, (0.1 pmol DNA)
100	6.5 ng
300	19.5 ng
500	32.5 ng
1000	65.0 ng
2000	130.0 ng
3000	195.0 ng
4000	260.0 ng
5000	325.0 ng

PROTOCOLS

A. LIC cloning

- Read the Important Notes section on p. 9 before starting.
- Mix the 5X LIC buffer by vortexing before each use.

1. To generate the necessary 5′ and 3′ overhangs on the purified PCR template, prepare the following reaction mixture at room temperature:

Component	Volume
5X LIC Buffer	2 µL
Purified PCR product	0.1 pmol
Water, nuclease-free	to 9 µL
T4 DNA Polymerase, 1u/µL	1 µL
Total volume	10 µL

   Vortex briefly and centrifuge for 3-5 s.

2. Incubate the reaction mixture at room temperature (20-25°C) for 5 min.
   Note. *Do not exceed 5 min.*
3. Stop the reaction by adding 0.6 µL of 0.5M EDTA, mix well.
   Note. *Store the prepared PCR product in the reaction mixture at -20°C if the annealing step with the LIC vector cannot be performed immediately. Thaw and mix carefully prior to performing the annealing reaction.*
4. Set up the **annealing reaction**:

   Add 1 µL pLATE, LIC-ready vector (60 ng, 0.02 pmol DNA) to the T4 DNA polymerase treated PCR product prepared in steps 1–3 of this protocol.

   Vortex briefly and centrifuge for 3-5 s.

5. Incubate the annealing mixture at room temperature (20-25°C) for 5 min.
   Note. *Annealing is complete within 5 min of incubation. Reactions can be incubated up to 2 hours without affecting results. Longer incubation times do not improve efficiency.*
6. Use the annealed mixture directly for bacterial cell transformation.

B. Transformation

- The aLICator LIC Cloning Kits are compatible with all common *E.coli* laboratory strains. See Tables 4 and 5 for more detailed recommendations.
- For successful cloning, competent *E.coli* cells should have an efficiency of at least 1×10^6 cfu/µg of supercoiled plasmid DNA. To check the efficiency, prepare a control transformation with a supercoiled vector DNA, such as pUC19 DNA, #SD0061 (Table 4).

Table 4. Expected transformation efficiency (control transformation).

Transformation method	Number of transformants per µg of supercoiled plasmid DNA	Amount of pUC19 DNA for control transformation (to yield ~ 1000 colonies per plate)	Volume of competent cells
TransformAid Bacterial Transformation Kit (#K2710)*	~1×10^7	0.1 ng	50 µL
Calcium chloride transformation	~1×10^6	1 ng	50 µL
Electroporation	~1×10^9	0.01 ng	40 µL

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

- Please refer to the table below for recommendations for bacterial cell transformation with the annealing mixture. The number of transformant colonies depends on the transformation efficiency of the competent cells.

Table 5. Recommendations for bacterial cell transformation with the annealing mixture.

Transformation method	Volume of the annealing mixture for transformation	Volume of competent cells for transformation	Volume of transformation mix to spread on the selection plate	Expected number of colonies per plate
TransformAid Bacterial Transformation Kit (#K2710)	1-2.5 µL	50 µL	1/2 of transformation mix	100-1000 cfu
Calcium Chloride Transformation	2-6 µL	50 µL	1/10 of transformation mix	100-1000 cfu
Electroporation	0.5-1 µL	40 µL	1/20 of transformation mix	>1000 cfu

C. Analysis of recombinant clones

During LIC cloning, only the desired products are formed by annealing of vector and insert. Therefore, normally more than 95% of transformants contain the desired insert in the correct orientation. Analyze 2-3 colonies for the presence of the DNA insert using one of the following methods:

Colony PCR

Colony screening by PCR can be used to confirm the presence of the insert DNA for cloned fragments shorter than 5 kb. For longer inserts, perform restriction digestion analysis.

1. Prepare sufficient PCR master mix for the total number of colonies to be analyzed. For each 20 µL reaction, mix the following reagents:

Component	Using *Taq* DNA Polymerase	Using 2X PCR Master Mix
10X *Taq* buffer **or** 10X DreamTaq Green Buffer	2.0 µL	–
dNTP mix, 2 mM each	2.0 µL	–
25 mM $MgCl_2$	1.2 µL	–
LIC Forward Sequencing primer, 10 µM	1 µL	1 µL
LIC Reverse Sequencing primer, 10 µM	1 µL	1 µL
Water, nuclease-free	12.7 µL	8 µL
Taq DNA Polymerase 5 u/µL, #EP0401 ***or*** DreamTaq Green DNA Polymerase 5 u/µL**,** #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 prepared mix into PCR tubes on ice.
3. Pick an individual colony and resuspend in 20 µL of the PCR prepared mix.
4. Perform PCR: 95°C, 3 min; 94°C, 30 s, 58°C, 30 s, 72°C 1 min/1kb; 25 cycles.
5. Analyze 10 µL of the PCR mixture on an agarose gel for the presence and size of the PCR product. PCR products obtained with LIC Forward and LIC Reverse Sequencing Primers should be 178 bp, 266 bp, 264 bp and 205 bp (plus insert size) for pLATE11, pLATE51, pLATE52 and pLATE31 vectors respectively.

Restriction analysis

Isolate plasmid DNA from an overnight bacterial culture. The GeneJET Plasmid Miniprep Kit (#K0502/3) can be used to rapidly recover high quality plasmid DNA. To digest DNA from recombinant clones in just 5 minutes, use Thermo Scientific FastDigest restriction enzymes (www.thermoscientific.com/onebio)

Sequencing

Use the LIC Forward Sequencing primer or LIC Reverse Sequencing primer supplied with the kit to sequence the cloned insert. *See* page 7 for primer sequences.

Protein expression and purification

After a recombinant plasmid DNA has been constructed and its sequence is confirmed, it can be used to transform a competent *E.coli* strain containing the T7 RNA polymerase gene under the control of the IPTG - inducible *lacUV5* promoter. *E. coli* expression host strains lysogenic for bacteriophage λDE3, such as BL21 (DE3), HMS 174 (DE3) or others may be used.

Note. *The basal expression level of pLATE vectors is very low; therefore it is not necessary to use lysozyme for T7 polymerase repression as recommended for other T7lac promoter containing vectors.*

General recommendations:

- For transformation of an expression host, use recombinant plasmids containing the desired insert in the correct orientation. The insert sequence and orientation can be confirmed by restriction digestion analysis, colony PCR or sequencing.
- Selected clones are grown overnight at 37ºC in LB medium containing 100 µg/mL ampicillin in shaking incubator, at 220-250 rpm/min.
- An overnight culture should be used to inoculate fresh LB/Amp medium (dilution ratio 1:50). Incubate at 37ºC in a shaking incubator (220-250 rpm/min) until the culture reaches an optical density OD_{600} of 0.5-0.6 (~2 h).
- To induce protein expression, add IPTG to a final concentration of 1 mM and incubate the culture at 37ºC in a shaking incubator (220-250 rpm/min) for 3 h.
- Harvest the culture by centrifugation.
- Affinity-purify His-tagged proteins following recommendations of your IMAC resin provider.
- If downstream applications for expressed protein require affinity tag removal, aLICator LIC Cloning and Expression Kit 2 or Kit 4, each containing a different protease recognition sequence, should be used. Enterokinase or WELQut Protease (Thermo Scientific, #KO0861) are companion products for these kits and should be purchased separately.
- WELQut Protease may be used during affinity purification of the target protein because of the built-in His tag and on-column proteolytic activity. After the purification step, the protein of interest is eluted with the flow-trough while the WELQut Protease and cleaved fusion tag remain bound to the resin in purification column. Detailed information and the protocol for WELQut Protease is enclosed in the product pack (#KO0861) or is available on www.thermoscientific.com/onebio.

CONTROLS

A. LIC cloning

A control cloning reaction using the appropriate 720 bp Control PCR fragment, included in the kit (nucleotide sequence is available in the product page on www.thermoscientific.com/alicator)can be used to assess the efficiency of the LIC reaction.

1. To generate the necessary 5′ and 3′ overhangs on the Control PCR fragment, prepare the following reaction mixture at room temperature:

Component	Volume
5X LIC Buffer	2 µL
Control PCR fragment for a specific pLATE vector	2 µL (0.1 pmol)
Water, nuclease-free	5 µL
T4 DNA Polymerase, 1u/µL	1 µL
Total volume	10 µL

   Vortex briefly and centrifuge for 3-5 s.

2. Incubate the reaction mixture at room temperature (20-25°C) for 5 min.

   Note. *Do not exceed 5 min.*

3. Stop reaction by adding 0.6 µL 0.5M EDTA and mix well.

   Note. *Store the prepared PCR product in reaction mixture at -20°C if annealing step with LIC vector will not be performed immediately. Thaw and mix carefully before the annealing reaction.*

4. Set up the **annealing reaction**:
   Add 1 µL pLATE, LIC-ready vector (60 ng, 0.02 pmol DNA) to the Control PCR fragment reaction mixture prepared in steps 1–3 of this protocol. Vortex briefly and centrifuge for 3-5 s.

5. Incubate the annealing mixture at room temperature (20-25°C) for 5 min.

   Note. *Annealing is complete within 5 min of incubation. Reactions can be incubated up to 2 hours without affecting results. Longer incubation times do not improve efficiency.*

6. Use 2.5 µL of the annealing mixture for transformation (*see* Table 5 on p. 13 for expected transformation efficiency).

Analyze colonies by colony PCR (*see* p. 14), or streak colonies on agar plates containing 100 µg/mL ampicillin and 30 µg/mL chloramphenicol –the cloned control insert codes for chloramphenicol acetyltransferase under control of the *tet* promoter so colonies containing the recombinant plasmid with control fragment will be resistant to this antibiotic. At least 9 out of 10 analyzed colonies should contain recombinant plasmid with the 720 bp insert and be resistant to chloramphenicol. 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 4 on page 13 for estimated control transformation efficiency.

B. Protein expression

The control pLATE31-Cm plasmid contains a cloned gene coding for chloramphenicol acetyltransferase (*cat*) under control of T7 promoter and serves as a positive control for induction of target protein expression. The *cat* coding sequence has been cloned via LIC procedure into the pLATE31 vector and encodes a C-terminal His-tag.

Protein expression induction in bacterial cultures containing the pLATE31-Cm plasmid should give rise to a prominent (~30 kDa) protein band when whole cell lysates are analyzed by SDS-PAGE.

1. Use 1 µL of pLATE31-Cm DNA to transform a preferred expression strain. Plate transformants on LB agar plates containing 100 µg/mL ampicillin.
2. Select clones and grow overnight at 37ºC in LB medium containing 100 µg/mL ampicillin in a shaking incubator (220-250 rpm/min).
3. Use overnight culture to inoculate fresh LB/Amp medium (dilution ratio 1:50). Incubate at 37ºC in a shaking incubator (220-250 rpm/min) until the culture reaches an optical density OD_{600} of 0.5-0.6 (~2 h).
4. To induce expression, add IPTG to a final concentration of 1 mM and incubate the culture at 37ºC for 3 h in a shaking incubator (220-250 rpm/min).

   Note. *Harvest 500 µL of culture by centrifugation and freeze at -20 ºC as an uninduced control.*

5. Harvest 250 µL of induced culture by centrifugation for SDS-PAGE analysis.

   A prominent ~30 kDa protein band should be visible in the gel lane containing induced cell lysate. PageBlue Protein Staining Solution (#24620) can be used to visualize proteins separated in 1D, 2D and IEF polyacrylamide gels.

APPENDICES

pLATE11 vector

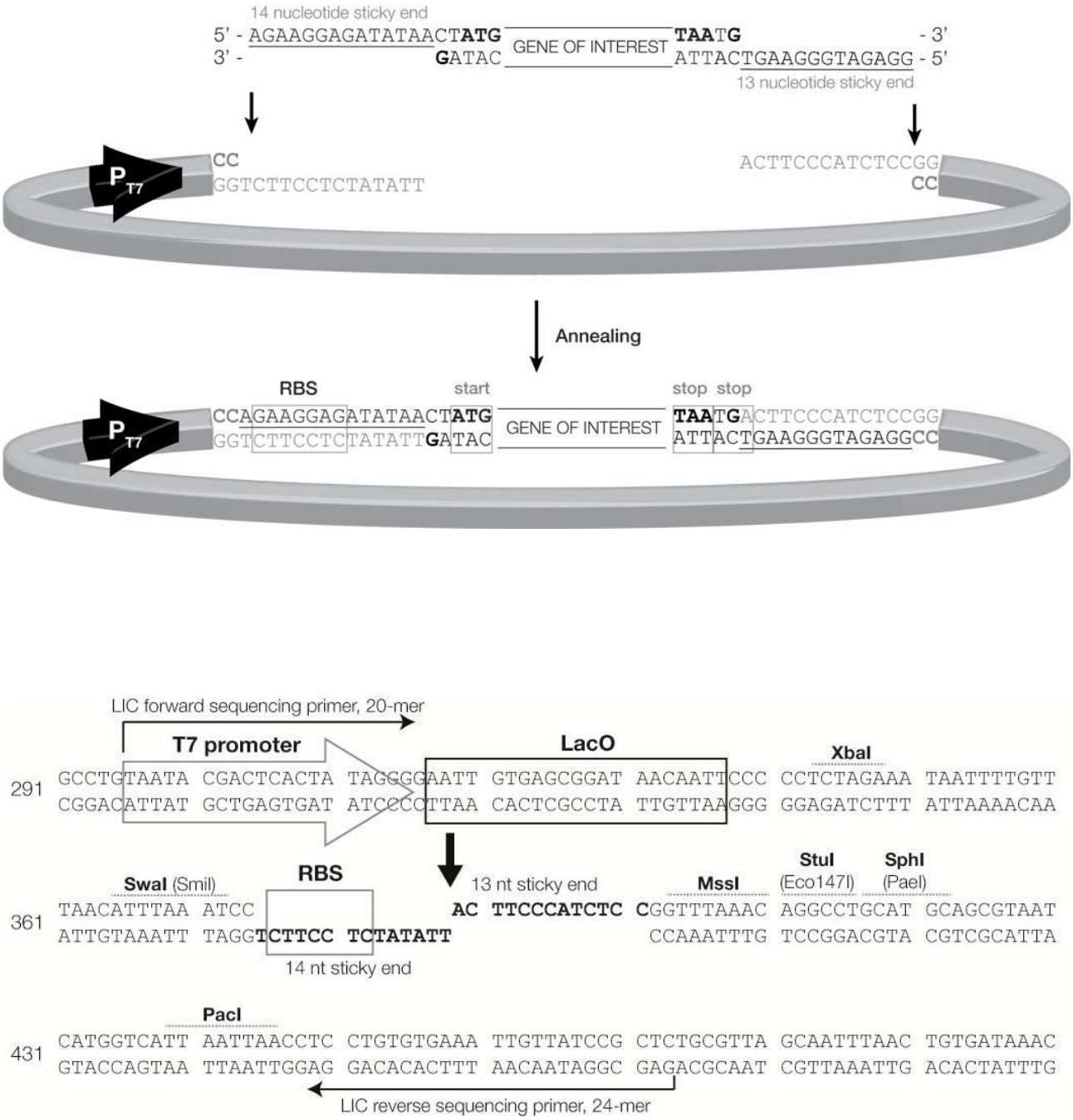

Figure 3. Diagram outlining the cloning strategy with the pLATE11 vector and resulting construct.

pLATE51 vector

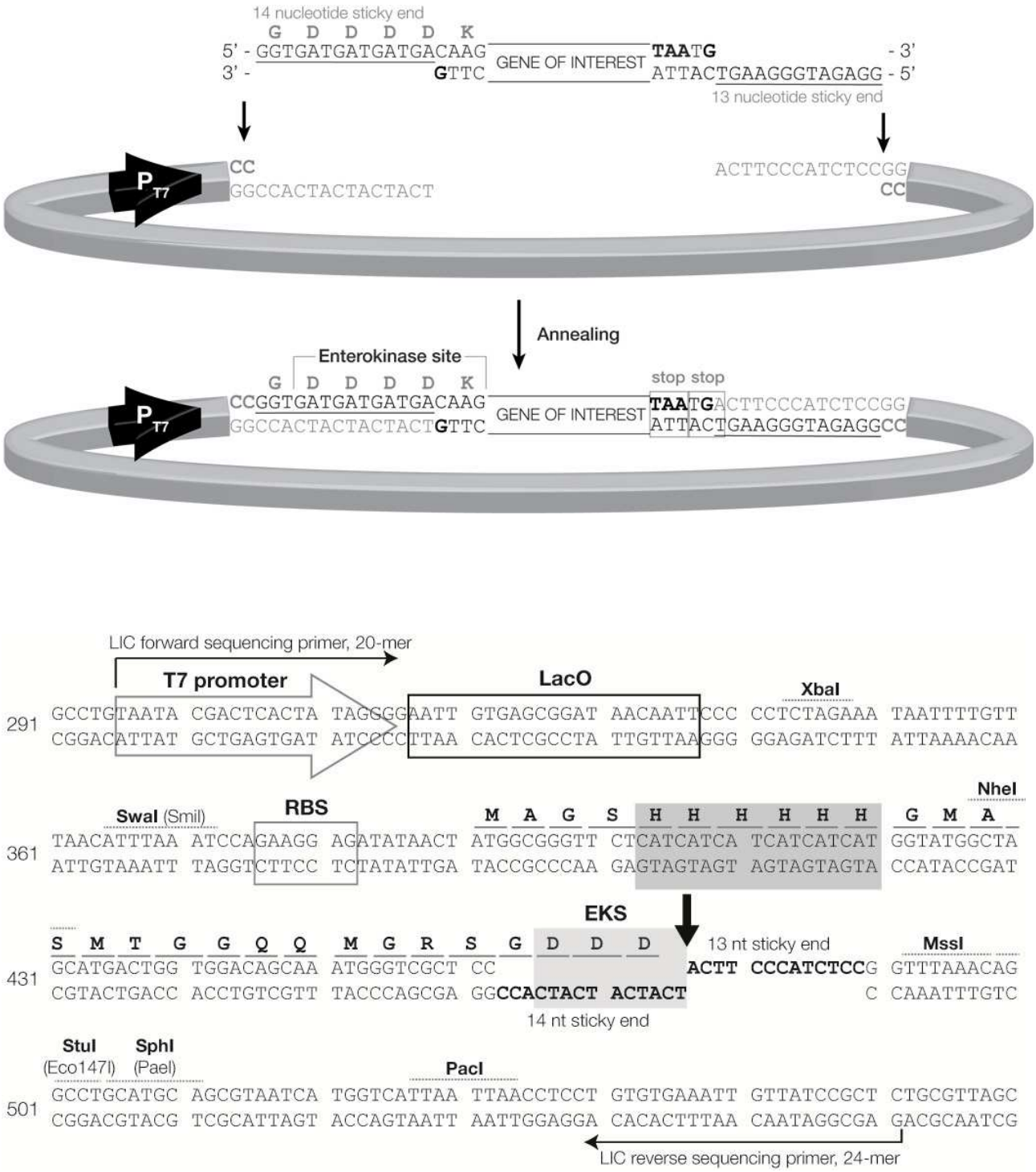

Figure 4. Diagram outlining the cloning strategy with the pLATE51 vector and resulting construct.

pLATE31 vector

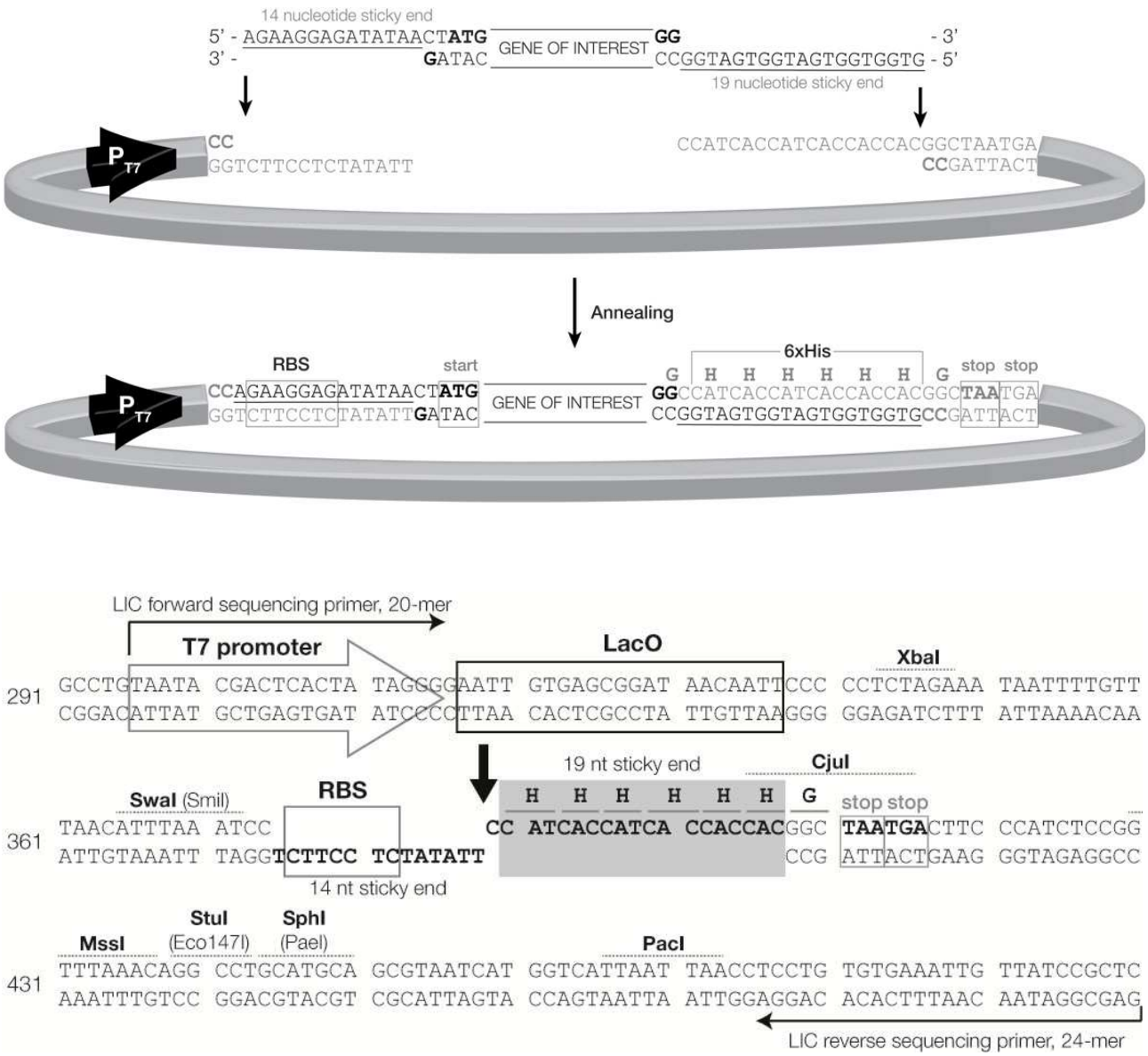

Figure 5. Diagram outlining the cloning strategy with the pLATE31 vector and the resulting construct.

pLATE52 vector

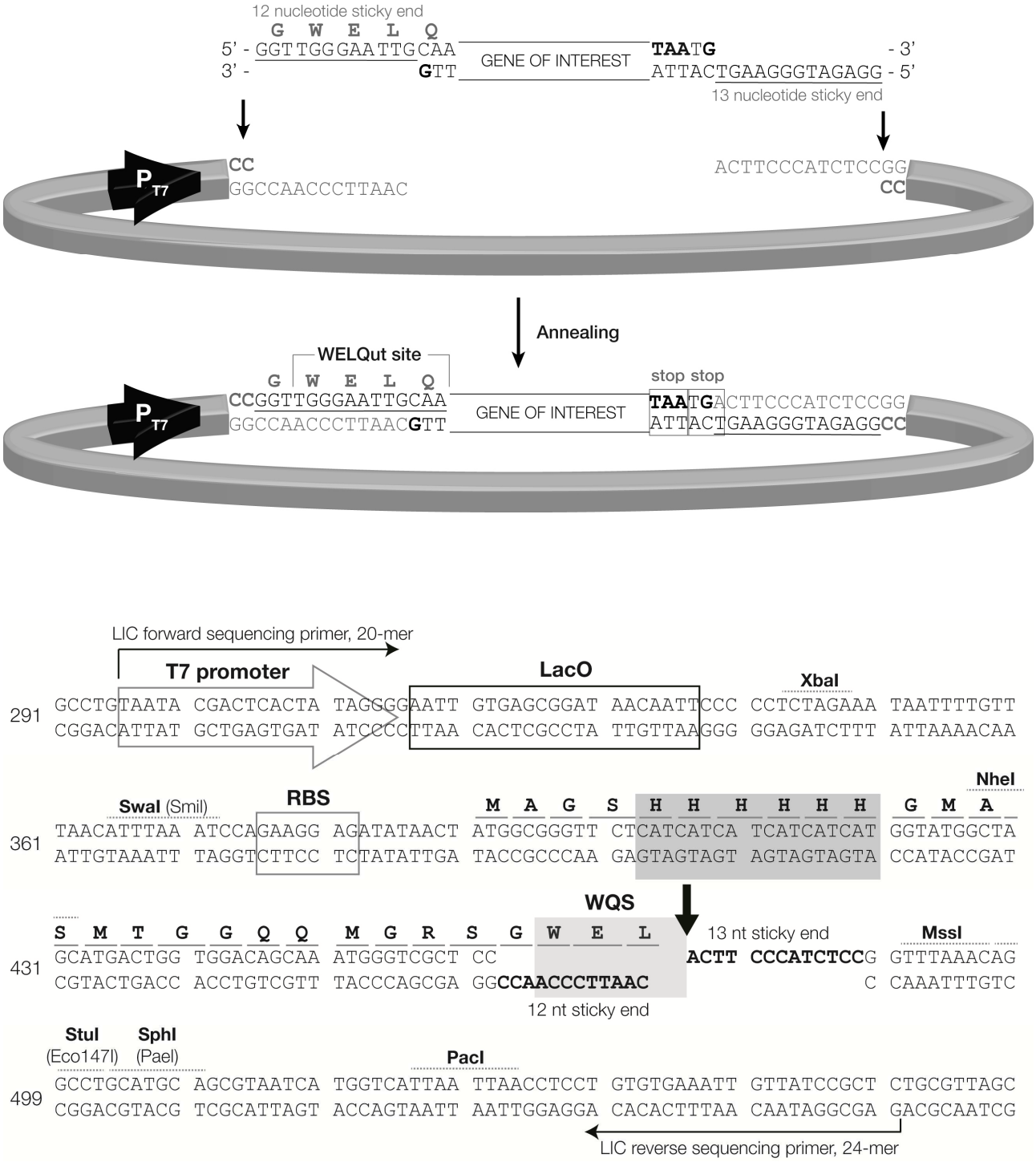

Figure 6. Diagram outlining the cloning strategy with the pLATE52 vector and the resulting construct.

RECIPES & SUPPLEMENTARY PROTOCOLS

Ampicillin stock solution (50 mg/mL)

1. Dissolve 0.25 g ampicillin sodium salt in 5 mL of deionized water.
2. Filter-sterilize and store in aliquots at 4°C.

Chloramphenicol stock solution (30 mg/mL)

1. Dissolve 0.3 g chloramphenicol in 10 mL of ethanol (96%).
2. Store in aliquots at 4°C.

LB Medium

1. To prepare LB 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. Autoclave.

LB-ampicillin plates

1. To 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. Allow the medium to cool to 55°C.
4. Add 2 mL of ampicillin stock solution (50 mg/mL) to a final concentration of 100 µg/mL.
5. Mix gently and pour plates.

LB-chloramphenicol plates

1. To prepare LB-agar Medium (1 liter), weigh out:
   Bacto Tryptone 10 g,
   Bacto Yeast Extract 5 g,
   NaCl 5 g.
1. Dissolve in 800 mL of water, adjust pH to 7.0 with NaOH and add water to 1000 mL.
2. Add 15 g of agar and autoclave.
4. Allow the medium to cool to 55°C.
5. Add 1 mL of chloramphenicol stock solution (30 mg/mL) to a final concentration of 30 µg/mL.
6. Mix gently and pour plates.

IPTG solution 100 mM

1. Dissolve 238.3 mg IPTG in 10 mL of deionized water.
2. Filter-sterilize and store in aliquots at -20°C.
   Or use ready-to-use IPTG solution 100 mM (#R1171).

TROUBLESHOOTING

Problem	Cause and Solution
Few or no transformants	**Low transformation efficiency.** Verify transformation efficiency with supercoiled plasmid DNA (e.g., pUC19). Refer to Table 4 on page 13 for expected control transformation efficiency values. **dNTPs were not completely removed from PCR product**. dNTPs in the PCR reaction mixture must be completely removed prior to T4 DNA polymerase treatment for successful generation of specific vector-compatible overhangs. Gel purify the PCR product using GeneJET Gel Extraction Kit, #K0691 or a similar method. **Incorrect quantity of insert DNA used for reaction with T4 DNA polymerase.** While the kit performs well with 0.05–0.2 pmol of PCR product, the optimal amount to use in the reaction with T4 DNA polymerase is 0.1 pmol of DNA. Refer to Table 3 on p. 11 or use dedicated software such as www.thermoscientificbio.com/webtools/reviewer/ to access formulas to determine pmol amounts. **The PCR product was damaged by exposure to 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, like crystal violet, to visualize DNA in ambient light (3, 4).
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 the addition of ampicillin.

Problem	Cause and Solution
Background colonies that contain plasmids with incorrect inserts	**PCR products are contaminated with a template that codes for ampicillin resistance.** Gel-purify the PCR product if template DNA encodes 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 treatment with T4 DNA polymerase. 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. Short DNA fragments (<1kb) are cloned with much higher efficiency than longer fragments. Therefore, long PCR products must be purified to remove any smaller fragments from the solution. Protect PCR products from both mechanical sharing and damage by nucleases: • Store PCR products at -20°C if they will not be used immediately in the LIC protocol. • Use clean labware, razor blades and electrophoresis tanks. Prepare fresh electrophoresis running buffer for the gel purification procedure.
Background colonies contain plasmids without inserts	In general greater than 95% of colonies contain the desired vector and insert. However; transformants can contain empty plasmids. Normally Calcium Chloride transformation using 60 ng of LIC vector gives 5–30 false positive colonies per plate
Sequence errors in the cloned insert	**PCR product was damaged by exposure to UV light during excision from an 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 plastic plate during UV illumination. Alternatively, use dyes, like crystal violet, to visualize DNA in ambient light (3, 4). **Low fidelity DNA polymerase used for PCR.** When a PCR product will be used in cloning applications, use a high-fidelity DNA polymerase with proofreading activity, such as Phusion® High-Fidelity DNA Polymerase (F530S/L) to ensure sequence accuracy. Follow the recommendations for high fidelity PCR as outlined by the manufacture of the polymerase. The likelihood of introducing PCR-generated mutations can be further reduced by using less than 25 PCR amplification cycles. **Errors in PCR primers.** Use HPLC-purified primers to decrease the possibility of primer-derived mutations. If the cloned PCR product contains sequence errors where the primer anneals and the same error persists in more than one clone, re-order the PCR primers from a reputable supplier and repeat the procedure starting from the PCR step.

Problem	Cause and Solution
Protein expression is low or absent	**Bacterial strain is not suitable for protein expression.** Ensure that the *E. coli* strain used for protein expression carries the gene encoding T7 RNA polymerase. Use only molecular grade (purity ≥99%) IPTG. Perform the control protein expression experiment according to instructions on p. 17. Optimize the expression system by assaying several suitable expression strains such as is BL21 (DE3), HMS174 (DE3) or ER2566. **Unstable protein**. Some proteins cannot be expressed as tagged fusion proteins or are more stable with either an N- or C-terminal tag. The stability and tag preference of a particular protein must be determined experimentally. **Rare tRNA codons** Most amino acids are encoded by several codons with each species having its own specific set of more frequently used codons. Codons used in heterologous genes might be underrepresented or completely absent in *E. coli* and cause translational frameshifts, premature termination, ribosome stalling or a significant slow-down in translation. To improve translation, use *E. coli* strains supplemented with plasmid-borne rare-codon tRNA genes or adapt specific gene sequences for expression in *E. coli.* **Protein expression is too low to be detected with Coomassie staining** If protein expression levels are too low to be detected by Coomassie staining, silver stain, Western blot or enzymatic assays can be used.
Insoluble protein	**Improper protein folding** Unnaturally high protein synthesis rates lead to missing disulfide bonds and improper protein folding which in turn leads to protein degradation or formation of inclusion bodies. In some cases this problem can be solved by lowering the induction temperature to 30º, 25 º or 20ºC or reducing the IPTG concentration to 0.1–0.5 mM while simultaneously increasing induction time to 5-10 hours.

References:

1. Studier F.W. and Moffatt B.A., Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol, 189:113-130, 1986.
2. Aslanidis C., de Jong P. J., Ligation-independent cloning of PCR products (LIC-PCR). Nucleic Acids Res., 18(20): 6069–6074, 1990.
3. 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.
4. Adkins, S., Burmeister, M., Visualization of DNA in agarose gels and educational demonstrations, Anal Biochem., 240 (1), 17-23, 1996.

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.thermoscientific.com/onebio for Material Safety Data Sheet of the product.