pDual Expression Vector

Instruction Manual

Catalog #214501 and #214502

Revision B

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214501-12

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pDual Expression Vector

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pDual Expression Vector

MATERIALS PROVIDED

Material provided	Concentration	Quantity
pDual expression vector[a]		
Catalog #214501	1 µg/µl	20 µg
Catalog #214502	1 µg/µl	100 µg
XL1-Blue host strain	—	500 µl

[a] The complete nucleotide sequence and list of restriction sites for the pDual vector is available at *www.genomics.agilent.com* or from GenBank® (Accession Number AF041247).

STORAGE CONDITIONS

pDual Expression Vector: –20°C
XL1-Blue Host Strain: –20°C

ADDITIONAL MATERIALS REQUIRED

Equipment

Thermocycler

Sterile Media and Reagents

Ammonium acetate
Ethanol
LB-kanamycin agar plates[§]
TE buffer[§]
Cloned *Pfu* DNA Polymerase
MRF´ Competent Cells (if *Eam*1104 I sites are present in insert)
*Eam*1104 I restriction enzyme
T4 DNA Ligase
5-methyldeoxycytosine (^{m5}dCTP)

NOTICE TO PURCHASER

The use of the CMV Promoter is covered under U.S. Patent Nos. 5,168,062 and 5,385,839 owned by the University of Iowa Research Foundation and licensed FOR RESEARCH USE ONLY. For further information, please contact UIRF at 319-335-4546.

[§] See *Preparation of Media and Reagents.*

Revision B

INTRODUCTION

The pDual expression vector directs expression of heterologous genes in both mammalian and prokaryotic systems. For constitutive expression in mammalian cells, the pDual expression vector contains a mutagenized version of the promoter/enhancer of the human cytomegalovirus (CMV) immediate early gene (see Table I and Figure 1). Inducible gene expression in prokaryotes is directed from the hybrid T7/*lacO* promoter; the pDual expression vector carries a copy of the lac repressor gene (*lacI*^{q}), which mediates tight repression in the absence of isopropyl-β-D-thio-galactopyranoside (IPTG). Efficient translation of mRNA generated in either the mammalian or prokaryotic system is achieved by a tandemly arranged Shine-Dalgarno[1]/Kozak[2] consensus sequence. In both bacterial and mammalian cells, the dominant selectable marker is the neomycin phosphotransferase gene which is under the control of the β-lactamase promoter in bacterial cells and the SV40 promoter in mammalian cells. Expression of the neomycin phosphotransferase gene in mammalian cells allows stable clone selection with G418, whereas in bacteria the gene confers resistance to kanamycin selection.

The unique cloning region of the pDual expression vector is characterized by the presence of two *Eam*1104 I recognition sequences (CTCTTC) directed in opposite orientations and separated by a spacer region encoding two *Eco*R I sites. Digesting the vector with the *Eam*1104 I restriction enzyme creates a 3-nucleotide 5´ overhang that is complementary to the translation initiation codon (ATG) of the DNA insert.

Inserts must be generated by PCR amplification with primers that contain *Eam*1104 I recognition sites and a minimal flanking sequence at their 5´ termini. The ability of *Eam*1104 I to cleave several bases downstream of its recognition site allows the removal of superfluous, terminal sequences from the amplified DNA insert. The elimination of extraneous nucleotides and the generation of unique, nonpalindromic sticky ends permit the formation of directional seamless junctions during the subsequent ligation to the pDual expression vector.[3,4]

The pDual vector contains the Calmodulin Binding Peptide (CBP) affinity tag, located 3´ to the cloning site, for optional fusion of the affinity tag to the carboxy terminus of the protein-coding sequence of interest. The CBP-affinity tag is preceded by a thrombin cleavage site which allows the removal of the fusion tag from the protein of interest.

TABLE I

Features of the pDual Vector

Feature	Nucleotide Position
bla promoter	2–126
SV40 promoter	146–484
neomycin/kanamycin resistance ORF	519–1313
HSV-thymidine kinase (TK) polyA signal	1487–1759
pUC origin of replication	1898–2565
lacI^{q} repressor ORF	2652–3611
CMV promoter	3810–4394
T7 promoter with *lac* operator	4399–4444
ribosome binding site	4461-4469
Kozak sequence	4470-4478
Eam1104 I site (reverse complement)	4480
EcoR I site	4485
EcoR I site	4523
Eam1104 I site	4528
thrombin target	4535–4552
calmodulin binding peptide (CBP)	4553–4630
5´-splice site	4637–4653
lac operator	4659–4686
SV40 3´-splice site	4732–4791
SV40 polyA signal	4801–5013
T7 terminator	5022–5065
f1 origin of ss-DNA replication	5203–5509

The pDual Vector

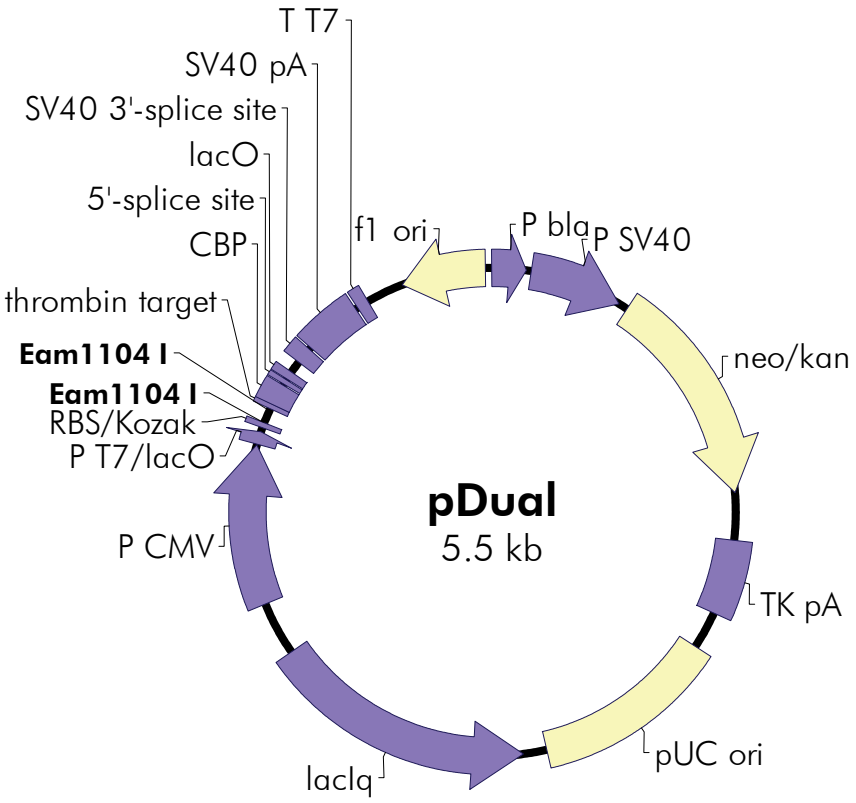

Figure 1 Map of the pDual expression vector. The complete nucleotide sequence and list of restriction sites for the pDual vector is available from *www.genomics.agilent.com* or from the GenBank® database (Accession #AF041247). The sequence provided at *www.genomics.agilent.com* has been verified for accuracy at the cloning junctions. The remainder of the sequence was compiled from existing data.

PROTOCOL CONSIDERATIONS

Insert Primer Design

1. The 5´ terminus of the primers must contain an *Eam*1104 I recognition site. *Eam*1104 I is a type IIS restriction enzyme that has the capacity to cut outside its recognition sequence (5´-CTCTTC-3´). The cleavage site extends one nucleotide on the upper strand in the 3´ direction and four nucleotides on the lower strand in the 5´ direction (see Figure 2). Digestion with *Eam*1104 I generates termini that feature three nucleotides in their 5´ overhangs.

2. A minimum of two extra nucleotides (N_{-1} and N_{-2} in Figure 2) must precede the 5´-CTCTTC-3´ recognition sequence in order to ensure efficient cleavage of the termini. The bases preceding the recognition site can be any of the four nucleotides.

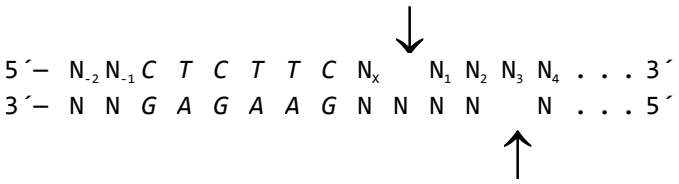

FIGURE 2 Restriction recognition sequence for *Eam*1104 I

3. The forward primer must be designed with one extra nucleotide (N) located between the *Eam*1104 I recognition sequence and the gene's translation initiation codon, in order to generate the necessary 5´-ATG overhang that is homologous to the vector sequence. The forward primer should be designed to look as follows:

   5´– N N C T C T T C N A T G $(X)_{15}$ – 3´

   where N denotes any of the four nucleotides and X represents gene-specific nucleotides.

4. The reverse primer must be designed with one nucleotide (N) located between the *Eam*1104 I recognition sequence and the AAG triplet which comprises the 5´ overhang that is homologous to the vector sequence. Depending on whether or not the CBP affinity tag is desired as a fusion partner, the reverse primer should be designed to look as follows:

   a. Reverse primer design that allows the expression of the CBP fusion tag:

   5´– N N *C T C T T C* N A A G $(X)_{15}$ –3´

   where N denotes any of the four nucleotides and X represents the gene-specific nucleotides.

   b. Reverse primer design that does not allow expression of the CBP fusion tag:

   5´– N N *C T C T T C* N A A G **C T A** $(X)_{12–15}$ –3´

   where N denotes any of the four nucleotides and X represents the gene-specific nucleotides. The necessary stop codons are shown in bold.

5. The primer should include a perfect template match of at least 15 nucleotides on the 3´ end of the PCR primer in addition to the *Eam*1104 I recognition sequence. The estimated T_m [$T_m \approx 2°C$ (A+T) + 4°C (G+C)] of the homologous portion of the primer should be 55°C or higher, with a G-C ratio of 60% or more.

pDUAL EXPRESSION VECTOR PROTOCOL

Digesting pDual Expression Vector with *Eam*1104 I

To generate a ligation-ready vector for PCR cloning, we recommend the following for digesting the pDual expression vector.

1. Digest ≤1 µg of the pDual expression vector using at least 24 units of the *Eam*1104 I restriction endonuclease.

   Note *For optimal cleavage of the vector, use 100 µg/ml BSA in the digestion reaction.*

2. Incubate the reaction at 37°C for 2 hours.

   Note *Dephosphorylation of the vector is not required because nonidentical, nonpalindromic sticky ends are generated by the type IIS* Eam*1104 I restriction endonuclease.*

3. Purify the digested vector using either of the two methods listed.

   - Gel purify the vector and resuspend in TE buffer (see *Preparation of Media and Reagents*).
   - Purify the vector using selective precipitation (see *Appendix I*).

   Note *If the selective precipitation method is used, the vector must be cut with* Eco*R I in addition to* Eam*1104 I before precipitating (to reduce background).*

Preparing PCR Amplified Insert

PCR Amplification of Insert

- To prevent mutations that could be introduced during PCR, use a high-fidelity polymerase, such as *Pfu* DNA polymerase, in the amplification reaction.
- To generate PCR products with vector compatible termini, *Eam*1104 I recognition sequences need to be present at the ends of each primer (see *Primer Design*).

- If the insert contains an internal Eam1104 I recognition site, the amplification reaction should be performed in the presence of 5-methyldeoxycytosine (^{m5}dCTP) for the last five cycles of the PCR. Incorporation of ^{m5}dCTP during the PCR amplification protects already-existing internal *Eam*1104 I sites from subsequent cleavage by the endonuclease. The primer-encoded *Eam*1104 I sites are not affected by the modified nucleotide because the newly synthesized strand does not contain cytosine residues in the recognition sequence.

  Note *The addition of the ^{m5}dCTP is delayed until the final five cycles of amplification to avoid the possible deamination of the ^{m5}dCTP due to extended exposure to heating and cooling cycles.*

Insert Purification

Before proceeding with the cloning protocol, carefully transfer the PCR products (from **below** the layer of mineral oil) to a fresh microcentrifuge tubes. The insert may be purified following several different methods [phenol:chloroform extraction, selective precipitation (see *Appendix I*: *Purifying the PCR Product by Selective Precipitation*), gel purification, spin-cup purification, or any other method of purification].

Note *Once the amplified insert has been purified, run an aliquot on an agarose gel to verify the success of the PCR amplification. (For PCR products <1 kb use 2% agarose. For PCR products >1 kb, use ≤1% agarose.)*

To prepare the insert for ligation, treat the insert with *Eam*1104 I (≥24 units).

Ligating Vector and Insert

The ligation can be performed either in the presence or absence of the *Eam*1104 I restriction endonuclease, depending on whether or not the vector and insert have been gel purified. In the event that the vector and insert are not gel purified, *Eam*1104 I (~6 units) should be present in the ligation reaction. Also, the final volume of the reaction should be increased to 20 µl to keep the glycerol content of enzymes ≤10% of the total reaction. Incubate the ligation reactions for 1 hour at room temperature or overnight at 16°C. Store the ligation reactions on ice until ready to use for transformation.

Note *If the experimental insert was amplified in the presence of methylated dNTPs, use XL1-Blue MRF' supercompetent cells.*

Transforming/Transfecting Ligated Vector/Insert

Transform the ligated product into appropriate competent cells.

Note *For the initial transformation, use an* end*A*⁻ *strain such as XL1-Blue supercompetent cells, XL10-Gold ultracompetent cells, or XL1-Blue MRF′ competent cells. Do not use BL21(DE3) competent cells, which are an* end*A*⁺ *strain.*

For a transformation protocol, see reference 5. Because the vector is kanamycin resistant, a long transformation protocol should be followed to reach an optimal expression level (incubate in SOC medium for 1 hour at 37°C before plating). Plate 5–10% of the transformed product on LB-kanamycin agar plates. Incubate the plates overnight at 37°C.

Note *For bacterial expression, transform mini-prep DNA into BL21(DE3) competent cells and plate 5–10% on LB-kanamycin agar plates. Select a colony and perform induction studies with and without Isopropyl-1-thio-β-D-galactopyranoside (IPTG) (50 μM–200 μM).*

Transfect the ligated product into appropriate mammalian cells. For a transfection protocol, see reference 5.

APPENDIX: PURIFYING THE PCR PRODUCT BY SELECTIVE PRECIPITATION

Selective precipitation purifies the vector and insert by removing excess PCR primers from the PCR product. In order to improve the overall cloning efficiency, We recommend selectively precipitating the PCR product regardless of the PCR enzyme used to generate the inserts as indicated in the following protocol.

1. Add an equal volume of 4 M ammonium acetate.
2. Add 2.5 volumes of 100% (v/v) ethanol equilibrated at room temperature.
3. Immediately spin the reaction tube in a microcentrifuge at 10,000 × *g* for 20 minutes at room temperature to pellet the DNA.
4. Carefully remove and discard the supernatant.
5. Wash the DNA pellet with 200 µl of 70% (v/v) ethanol.
6. Spin the reaction tube in a microcentrifuge at 10,000 × *g* for 10 minutes at room temperature. **Carefully** remove the ethanol with a pipet.
7. Dry the DNA pellet under vacuum.
8. Resuspend the DNA to the original volume using TE buffer.
9. Measure the optical density of the sample at an absorbance of 260 nm (OD_{260}) to determine the concentration of the vector or PCR product.
10. Store the purified vector or PCR product at 4°C until ready for further use.

TROUBLESHOOTING

Observation	Suggestion(s)
Little or no PCR product is observed on the agarose gel	The PCR primers have been designed incorrectly. Review *Primer Design*
	The PCR extension cycle is too short. Ensure a minimum extension time of 2 minutes/kb of the PCR target
The cloning efficiency or transformation efficiency is low as evidenced by few or no colonies forming after plating	The PCR product is impure. Purification of the PCR product is required before proceeding with ligation and transformation
	The digestion of the pDual expression vector is incomplete, or the pDual expression vector is impure. Verify that the pDual expression vector is completely digested and purified before continuing to ligation or transformation
	The concentration of the antibiotics in the agar plates is incorrect. Verify the correct antibiotic concentration and replate the transformation reactions

PREPARATION OF MEDIA AND REAGENTS

TE Buffer
10 mM Tris-HCl (pH 7.5)
1 mM EDTA

10× Ligase Buffer
500 mM Tris-HCl (pH 7.5)
70 mM $MgCl_2$
10 mM dithiothreitol (DTT)
Note *rATP is added separately in the ligation reaction*

LB Agar (per Liter)
10 g of NaCl
10 g of tryptone
5 g of yeast extract
20 g of agar
Add dH_2O to a final volume of 1 liter
Adjust the pH to 7.0 with 5 N NaOH
Autoclave
Pour into petri dishes (~25 ml/100-mm petri dish)

LB–Kanamycin Agar (per Liter)
Prepare 1 liter of LB agar
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml-filter-sterilized kanamycin
Pour into petri dishes (~25 ml/100-mm plate)

LB Broth (per Liter)
10 g of NaCl
10 g of tryptone
5 g of yeast extract
Add deionized H_2O to a final volume of 1 liter
Adjust to pH 7.0 with 5 N NaOH
Autoclave

LB–Kanamycin Broth (per Liter)
Prepare 1 liter of LB broth
Autoclave
Cool to 55°C
Add 5 ml of 10-mg/ml-filter-sterilized kanamycin

REFERENCES

1. Shine, J. and Dalgarno, L. (1974) *Proc Natl Acad Sci U S A* 71(4):1342-6.
2. Kozak, M. (1986) *Cell* 44(2):283-92.
3. Padgett, K. A. and Sorge, J. A. (1996) *Gene* 168(1):31-5.
4. Padgett, K. A. and Sorge, J. A. (1996) *Strategies* 9(1):14-16.
5. Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G. *et al.* (1987). *Current Protocols in Molecular Biology*. John Wiley & Sons, New York.

ENDNOTES

MSDS INFORMATION

Material Safety Data Sheets (MSDSs) are provided online at *http://www.genomics.agilent.com*. MSDS documents are not included with product shipments.